STRESS AND INFLAMMATION REGULATE THE *KYNURENINE PATHWAY* DIOXYGENASES IN A GLIA- AND TISSUE-SPECIFIC MANNER

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Neuroscience
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2017

Urbana, Illinois

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ABSTRACT

The inflammation and stress responses are intimately related adaptive responses to pathogens or other stressors upsetting our body's homeostasis. However, when infections or other stressors persist-or are sufficiently traumatic-our normally adaptive responses become maladaptive compromising our immune system and mental wellbeing. Depression accounts for ~33% of debilitating mental illness worldwide. Recently, studies have outlined mechanisms by which stressors inducing proinflammatory cytokines or glucocorticoids precipitate symptoms of depression. Central to this hypothesis are the glucocorticoid- and cytokine-inducible enzymes tryptophan and indoleamine 2,3-dioxygenases (DOs: Tdo2, Ido1 and Ido2). The DOs are rate-limiting for tryptophan metabolism by the *Kynurenine Pathway*. While accumulating evidence supports an interconnection between symptoms of depression and increased *Kynurenine Pathway* activity, the cellular specificity underpinning DO regulation by stress and inflammation remain poorly defined. Thus, (Chapter 1) after a review of the relevant literature, this thesis probes the regulation of DO expression (Chapter 2) by acute stress, and (Chapter 3) by peripheral inflammation and glucocorticoids, within the mouse brain, astrocytes, microglia, and peripheral tissues. We identify a unique role for astrocytes in the DO response to stress, and describe unique regulation patterns for astrocytes and microglia in the DO response to inflammation and glucocorticoids. Finally, using Cre-lox mediated cell-specific knockdown of Ido1 in mice, we probe the contributions of myeloid- and neuron-derived Ido1 to inflammation-induced anhedonia-like behavior reveling that myeloid-derived Ido1 contributes to inflammation-induced anhedonia. Together, the data highlight glia- and tissue-specific DO regulation by stress and inflammation, and cell-specific contributions of Ido1 to inflammation-induced anhedonia-like behaviors in mice.

For the love of my life…

my lovely Wife

ACKNOWLEDGMENTS

I cannot begin to express the depth of gratitude and appreciation I have for the years of support I have received from my wife, Victoria Dostal. After knowing each other for less than 2 years, you moved across the nation with me. I can never repay you for the birthdays, graduations, funeral celebrations, and other precious moments that passed us by as you stood by my side. You are my home away from home—you are my everything!

I am blessed for being raised in a family which greatly values education of both the mind and spirit. My father, Federico Dostal, a physician of internal medicine, practiced in Texas for 30 years and was beloved by our community. He always pushed me to excel academically. My mother, Edna Dostal (a medical technologist, chemical engineer, doctor of analytical chemistry, and registered nurse) never learned how to stop studying, and I took "The Road Not Taken" because of the wisdom of my mother's council. Nonetheless, her education only begins to highlight the inspiration my mom has given me and many others. Indeed, in this world seemingly full of only fleeting happiness, it is her devotion to our Catholic faith and her profound love of Jesus Christ that constantly redirects me on my journey to eternal joy.

Finally, I thank Bob M^{C}Cusker, to whom I am most grateful for introducing me to the love and labor of Science. It took 2-3 months of quick retorts and annoyed glances from him for me to finally stop calling him Dr. M^{C}Cusker—"It's just Bob". Thank you for always having your door open to us, and for sharing your years of experience in proposing, designing, executing, documenting, and publishing experiments in science. Thank you for being my advisor and mentor, and for always treating us like colleagues. I hope to be as fair, patient, and understanding of an advisor as you have been.

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CHAPTER 1: Introduction

1.1 Stress and stressors

In 1936, the Austrian-Canadian physician Hans Selye began to convince the world of his understanding of stress [1]. Dr. Selye is the '*father of stress*' and borrowed the term, in analogy, from physics and material science which define stress (force-per-unit-area) as proportional to strain (or a percent-change-in-length). Thus, physicists and engineers investigate stress in solid materials within the context of applied or inherent strains. Likewise, stress biologists investigate *biological stress* within the context of applied or inherent *stressors*. In Selye's own words, "This distinction between stressor and stress was perhaps the first important step in the scientific analysis of that most common biologic phenomenon that we all know only too well". Even so, often the term 'stress' is applied loosely and imprecisely outside of the stricter definitions applied within the discipline of stress biology [2]. This is a likely consequence of the explosive growth (Figure 1) of research into stress biology.

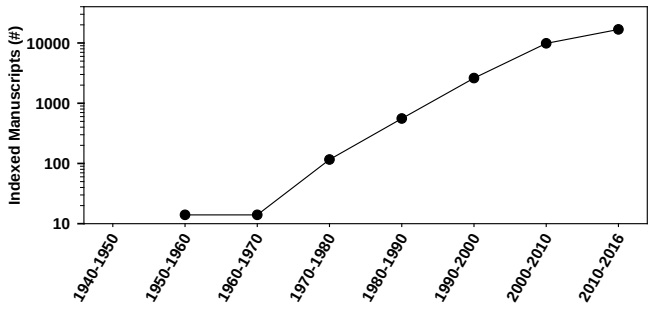

Figure 1. PubMed indexed manuscripts containing "Stress Response"

Stress results from homeostatic perturbations, or stressors, which activate the hypothalamic-pituitary-adrenal (HPA) axis and stimulate the immune system [3]. Indeed, the "stress response" is classically described by a fast sympathetic nervous system response mediated primarily by neurotransmitter action, followed by a relatively slow endocrine HPA axis response mediated primarily by endogenous glucocorticoid action [3]. Importantly, the

stress response is normally adaptive and necessary for survival; it facilitates adaptation to external stressors like shifts in ambient temperature [1] and prepares us for *fight or flight* [3]. Stressful situations alter behavior, with even acute stressors resulting in changes in cognition [4], [5]. The stress response also involves precise regulation of neuronal plasticity [3], neurogenesis [6], and changes in the central and peripheral immune-physiology [7].

1.2 Inflammation and Stress

The stress response is associated with immunological changes both in the peripheral and central nervous systems [7]. In men for instance, the sensitivity of their peripheral immune cells to the anti-inflammatory action of glucocorticoids is increased following an acutely stressful situation [8]. Moreover, norepinephrine, the primary neurotransmitter of the sympathetic nervous system, can augment immune cell activity [9]. By contrast, stimulation of the vagus nerve, the primary efferent nerve of the parasympathetic nervous system, has anti-inflammatory effects [10]. Thus, the stress response can modulate the immune system response by way of direct neuronal and indirect HPA axis (glucocorticoid) mediated pathways.

The immune system and the stress response are intricately and bidirectional related. For instance, glucocorticoid activity is necessary to control the pro-inflammatory response following infection [11], and absence of glucocorticoids is associated with transient increases in pro-inflammatory cytokines [12]. By contrast, psychological stress [13], [14] or glucocorticoids [15]–[17] can actually be sensitizing so that a subsequent immune challenge is associated with an exacerbated immune response. Indeed, timing of glucocorticoid administration relative to an immune challenge can determine the nature of the cytokine response [17]. For example, glucocorticoid exposure 2 h *before* an immune challenge potentiates pro-inflammatory

cytokine expression in brain and liver, whereas glucocorticoid exposure 1 h *after* LPS suppresses the same LPS-induced responses [14], [18], [19].

Acute stress results in increased brain expression of inflammatory mediators such as IL-1β [20] TNFα [21], [22] and iNOS [23]. While both the HPA axis and autonomic nervous system activity contribute to the increases in peripheral and central pro-inflammatory mediators following stress [7], [24], the proinflammatory cytokines IL-1β[25], TNFα[26], and IL-6[27] administered systemically also activate the HPA axis. The mechanisms by which these inflammatory mediators might regulate the stress response (or vice versa) is fascinating but unfortunately out of the scope of the current work. Nonetheless, how the stress-response and inflammatory mediators might regulate the *Kynurenine Pathway* is of significant relevance.

1.3 Stress, Inflammation and the *Kynurenine Pathway*

The indoleamine and tryptophan 2,3-dioxygenases (DOs: Ido1, Ido2 and Tdo2) are rate-limiting enzymes of the *Kynurenine Pathway* [28]–[31]. Tryptophan is metabolized via the *Kynurenine Pathway* for the production of NAD (Figure 2) [32]. Murine models of acute [33], [34] and chronic [34], [35] immunogenic sickness also exhibit increases in the *Kynurenine Pathway* activity. Likewise, stress increases brain [36]–[38] and plasma [36], [37], [39] kynurenine levels.

Cells in the periphery (i.e. macrophages and dendritic cells) and in the brain (i.e. microglia, neurons, and astrocytes) expressing the DOs can metabolize tryptophan to kynurenine; however, kynurenine itself may not be neuromodulatory and is further metabolized, with cell-specific precision, into downstream neuroactive metabolites (or kynurenines, i.e. Kyns) [32]. For example, astrocytes mainly produce kynurenic acid (KynA),

which can be neuroprotective, while microglia and macrophages are enzymatically equipped to produce more neurotoxic 3-hydroxykynurenine (3-HK) and quinolinic acid (QuinA) (Figure 2) [32], [40].

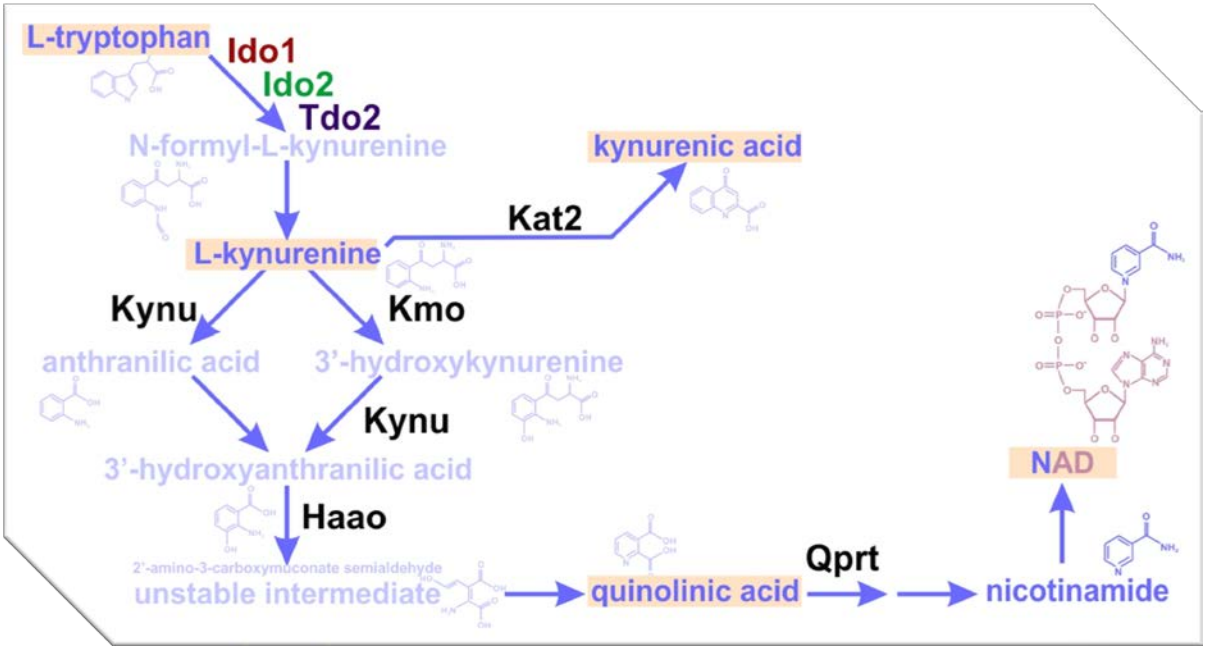

Figure 2. The *Kynurenine Pathway* of tryptophan metabolism. Tryptophan is an essential amino acid and therefore required from the diet. Greater than 90% of dietary tryptophan is metabolized via the *Kynurenine Pathway*.

Astrocytes uptake Trp and Kyn [41] producing KynA [42], [43] which modulates cholinergic [44], [45] and glutamatergic [46], [47] synaptic transmission. Nonetheless, a balance of neurotoxic and neuroprotective kynurenines is evidently essential, as a hallmark of Schizophrenia is increased saliva, plasma, CSF and brain levels of KynA [48], [49]. Moreover, a KMO allele resulting in reduced KMO expression is associated increased CSF levels of KynA and psychotic features in bipolar disorder [50]. Although endogenous levels of KynA are possibly below those required for direct NMDA-R inhibition at the glycine site [47], KynA activates presynaptic neuronal α7nAChRs to reduced glutamate release [46]. KynA levels also modulate cortical acetylcholine release, presumably via α7nAChRs [44]. Kat2^{KO} mice have 71% lower KynA in brain relative to WT mice, which was associated with increased glutamate levels and better

cognitive performance [47]. Nonetheless, although increased KynA may be associated with conditions such as bipolar disease and schizophrenia, increases in the microglial kynurenine QuinA [51] is more often associated with depression [52]. As such, Kmo^{KO} and $Haao^{KO}$ mice are protected from some inflammation-induced depression-like behaviors [53]. Even so, whether increased KynA, QuinA, or increases in other Kyns mediate psychiatric symptoms is unknown, and as likely a scenario maybe that an *imbalance* in these Kyns also precipitate psychiatric symptoms by altering neurotransmission [54]. Nevertheless, the DOs are rate-limiting for the production of Kyns and their cell-type specific regulation in the brain remains ill defined.

Of the DOs, Tdo2 is abundantly expressed in the liver relative to the brain [55] and hepatic Tdo2 mRNA is upregulated by acute stress [38], [56] or glucocorticoids [57]. Shimazu first reported increased hepatic Tdo2 activity (*in vitro* conversion of Trp→Kyn by liver homogenate) following peripheral administration of tryptophan, corticosterone, or following electric stimulation of the hypothalamic sympathetic nucleus [58]. Also, addition of dexamethasone (DEX, a synthetic glucocorticoid) to primary hepatocytes increases Tdo2 activity [59], [60] and Tdo2 mRNA [61], [62] expression. Thus, glucocorticoid regulation of hepatic Tdo2 activity [63]–[65] is well accepted, and only recently has brain Tdo2 been associated with stress-induced depression-like behaviors [56]. Nonetheless, the cell-types expressing in the brain that respond to stress or inflammation with increased Tdo2 activity are poorly defined.

Inflammation [33], [66]–[68] and stress [69]–[71] induce Ido1-dependent depression-like behaviors. Inflammation-induced cytokines or stress-induced glucocorticoids promote depression-like behaviors such as increased immobility during the tail suspension (TST) or forced swim (FST) tests [33], [69], [71], [72], decreased sucrose preference [68], [71], [72] and diminished cognitive function [67], [72]. Following the intracerebroventricularly (*i.c.v.*)

administration of lipopolysaccharide (LPS, endotoxin, an immune system activator), Ido1 mRNA and mRNA for pro-inflammatory mediators tumor necrosis factor (TNF)α, interleukin (IL)-6, and inducible nitric oxide synthase (iNOS) are significantly unregulated in the hippocampus, but central interferon-(IFN)γ (a type II INF) mRNA is not detected [73], [74]. Likewise, Fujigaki *et al.* reported peripheral Ido1 induction by LPS largely depended on TNFα, and that both IFNγ-dependent and independent pathways exist [75]. LPS incubation increases Ido1 expression and enzymatic activity in primary microglia [76], and in organotypic hippocampal slice cultures (OHSC) [73], by time-dependent, and dose-dependent and IFNγ-independent mechanisms. These results are in contrast to the notion that IFNγ is a principal inducer of Ido1 [30], [77]. More importantly, since we know that kynurenine is transported across the blood-brain barrier [78], cellular origins of inflammation or stress-induced increases in *Kynureinine Pathway* activity within the brain remain poorly defined.

There are considerable fewer studies investigating Ido2 than there are for Ido1. There is greater homology between human and mouse Ido2 proteins (84% similar) than human and mouse Ido1 proteins (77% similar) [79]. Ido2 is enzymatically active with 20% to 55% lower enzymatic activity than Ido1 [80], [81]. However, inhibiting Ido2 (siRNA) does not affect Ido1 expression but still suppressed NAD production inducing apoptosis in melanoma cells [82]. Nonetheless, naïve $Ido1^{KO}$ mice have lower hepatic Ido2 expression, but $Ido2^{KO}$ mice have normal hepatic Ido1 expression [83]. This effect is certainly tissue-specific as the same study demonstrates that neither $Ido1^{KO}$ nor $Ido2^{KO}$ impacts the expression of the remaining Ido in spleen [83]. Indeed, there are several reports of perturbed Ido2 expression and inducibility in $Ido1^{KO}$ mice [84]–[86]; therefore, the role of Ido2 in mediating what are largely considered Ido1-dependent behaviors is likely underappreciated.

While only scantily covered within this thesis, the *Kynurenine Pathway* has other important functions aside from modulating neurotransmission. It is the major pathway for generating nicotinamide for NAD synthesis. Indeed, it is likely that plasma kynurenine is elevated by inflammation due to an increased demand of NAD for poly(ADP-ribosyl)ation mediated repair of DNA damage caused by reactive oxygen species (ROS) [87], [88]. Inflammation-induced increases in plasma kynurenine are likely derived from multiple tissues. In fact, kynurenine is also produced by IFNγ stimulated endothelial cells where it also acts as a vasodilator [89], and LPS-induced hypotension is less prominent in Ido1^{KO} mice [89]. Ido1-mediated immune suppression occurs via both enzymatic and non-enzymatic mechanisms [90]. Enzymatically, dendritic cells (DCs) upregulate Ido1 in response to IFNγ [91] or CTLA-4 [92] to deplete Trp in the cellular microenvironment resulting in suppressed T-Cell proliferation. DC activation of regulatory T-Cells (Tregs) is also Ido1-dependent, and Tregs also suppress T-Cell proliferation [93]. Ido1 also suppresses the anti-tumor response by producing kynurenine which activates the aryl hydrocarbon receptor (AhR) [94]. Thus, Ido1 blockade is an active areas of cancer research [95]. Ido1 enzymatic activity of hepatic stellate cells can induce Treg expansion via the T-cell AhR [96]. Establishing immune tolerance to LPS requires AhR activation, as well as Ido1 enzymatic activity and signaling [97]. DCs treated with TGFβ also promote Ido1-depenent T regulatory cell (Treg) expansion independently of Ido1 enzymatic activity [90]. Ido1 encodes two tyrosine-based inhibitory motifs (ITIMs) that can directly interact with SOCS3 to mediate ubiquitin-proteasome targeting of Ido1 [98]. These Ido1 ITIMs are phosphorylated in DCs following TGFβ, but not IFNγ treatments [90]. As opposed to Ido1, Ido2 has only one ITIM and is not targeted for SOCS3-mediated ubiquitination like Ido1 [99]. Thus, these studies suggest that Ido1 is generally immune suppressive by enzymatic and non-enzymatic pathways, and that Ido2

may have limited signaling potential by virtue of key Ido1 sequences not conserved. Ido2 also contributes to cancer pathogenesis independently of Ido1. Small interfering RNA (siRNA) knockdown of Ido2 arrested tumor growth while increasing ROS and cell apoptosis [82]. The results of specific siRNA-mediated Ido2 knockdown were reversible by exogenous NAD [82]. Since Ido1 expression was not disrupted by the siRNA, this demonstrated an Ido1-independent mechanism for Ido2 in cancer pathogenesis [82] further demonstrating their non-redundant function. Flow cytometry analysis of spleen cells from WT and $Ido2^{KO}$ animals found no differences in leukocyte counts [100], but $Ido2^{KO}$ mice have attenuated autoimmune arthritic disease, lower auto-antibody production, diminished CD4+ helper T-cell (Th) populations, and Th cytokine production compared to arthritic WT mice [83]. Indeed, mice treated with an antibody targeting the Ido2 protein also have inhibited autoimmune arthritis reactions relative to controls [101]. Taken together, these studies not only demonstrate the non-redundant physiology of Ido1 and Ido2, but also suggest that Ido2 may be generally more pro-inflammatory, as opposed to Ido1 [30], [102].

During acute stress [103] and infection [104], [105], leukocyte reservoirs in bone marrow and spleen are mobilized. These immune cells traffic to multiple organs including the lung [104], liver and brain [105] where they may contribute to DO expression. While saline perfusion removes most monocytes from the lung tissue of animals treated with LPS [104], the net effects of cell trafficking on tissue-level DO expression is unrealizable without a more in-depth investigation. Nonetheless, inflammatory mediators and glucocorticoids are known regulators of the rate-limiting DOs; defining and contrasting DO regulation in the periphery and brain may ultimately contribute to our understanding of the pathogenesis depression within the context of increased inflammation or perceived stress. Clearly, understanding cell-type

specific contributions to tissue level DO expression is essential. Here in we describe the astrocyte and microglia role played in the brains DO response to acute stress (Chapter 2), glucocorticoids and inflammation (Chapter 2), as well as behavioral effects of microglia and neuron derived Ido1 on inflammation induced depression-like behavior (Chapter 3).

CHAPTER 2: Glia- and tissue-specific regulation of *Kynurenine Pathway* dioxygenases by acute stress of mice [1]

2.1 Abstract

Stressors activate the hypothalamic-pituitary-adrenal (HPA) axis and immune system eliciting changes in cognitive function, mood and anxiety. An important link between stress and altered behavior is stimulation of the *Kynurenine Pathway* which generates neuroactive and immunomodulatory kynurenines. Tryptophan entry into this pathway is controlled by rate-limiting indoleamine/tryptophan 2,3-dioxygenases (DOs: Ido1, Ido2, Tdo2). Although implicated as mediating changes in behavior, detecting stress-induced DO expression has proven inconsistent. Thus, C57BL/6J mice were used to characterize DO expression in brain-regions, astrocytes and microglia to characterize restraint-stress-induced DO expression. Stress increased kynurenine in brain and plasma, demonstrating increased DO activity. Of three Ido1 transcripts, only Ido1-v1 expression was increased by stress and within astrocytes, not microglia, indicating transcript- and glial-specificity. Stress increased Ido1-v1 only in frontal cortex and hypothalamus, indicating brain-region specificity. Of eight Ido2 transcripts, Ido2-v3 expression was increased by stress, again only within astrocytes. Likewise, stress increased Tdo2-FL expression in astrocytes, not microglia. Interestingly, Ido2 and Tdo2 transcripts were not correspondingly induced in Ido1-knockout ($Ido1^{KO}$) mice, suggesting that Ido1 is necessary for the central DO response to acute stress. Unlike acute inflammatory models resulting in DO induction within microglia, only astrocyte DO expression was increased by acute restraint-

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[1] Edited from original publication with headings, figures, and tables renumbered:
Dostal CR, Carson Sulzer M, Kelley KW, Freund GG & M^{C}Cusker RH. Glial and tissue-specific regulation of *Kynurenine Pathway* dioxygenases by acute stress of mice. *Neurobiology of Stress.* 7, 1-15 (2017).

stress, defining their unique role during stress-dependent activation of the *Kynurenine Pathway*.

2.2 Introduction

An acute stress response is necessary for survival; it facilitates adaptation to external stressors and primes the body for the metabolic, physical and cognitive demands of *fight-or-flight* [3]. Although classically assessed by HPA axis and sympathetic nervous system (SNS) activation, the stress response also involves precise changes in neuronal plasticity [3] along with central and peripheral immune activation [7], [24]. When the behavioral response to physical, psychological or metabolic stressors becomes maladaptive, profound consequences upsetting physical health and mental wellbeing occur [106]. Nearly half of Americans report experiencing at least one psychiatric disorder at some point in their lives, the most common being depression [107], [108]. Mounting evidence supports a causal link between stress-induced activation of the *Kynurenine Pathway* and psychiatric disorders, including depression [109]–[111] and schizophrenia [48], [112]. Stress can also trigger depression, as well as influence the length and severity of depressive episodes [113]. Similarly, stress is a comorbid factor for schizophrenia [114]. Thus, advancing our understanding of the mechanism(s) by which the brain responds to stress serves to elucidate the biology underpinning stress-related psychiatric disease.

Tryptophan (Trp) metabolism via the *Kynurenine Pathway* is initiated by three rate-limiting dioxygenases, DOs [32]. Acute predatory stress [115], foot shock [37] and physical restraint or immobilization [56] increase DO activity and mRNA expression in the brain and liver. The general dogma is that glucocorticoids regulate Tdo2 expression, while Ido1 and Ido2 are regulated by inflammatory mediators [32], [54]. Indeed, early work on stress and the

Kynurenine Pathway focused on the activation of hepatic Tdo2 (a.k.a. tryptophan pyrrolase) associated with HPA axis activation [58], [116]–[118]. Acute restraint-stress increases hepatic Tdo2 mRNA expression and activity [56] mediated in part by adrenocortical secretions [117]. Acute stress also increases Ido1 expression in the brain [39], [119] and periphery [39]. Nevertheless, there is only one report of stress regulating Ido2 expression in the CNS [120], although its unique role in immunophysiology is established [100].

Stress is associated with immunological changes both in the periphery and CNS [7], [121]. Acute stress increases brain cytokine levels including TNFα[21], [22] and IL-1β[20], [122]. These cytokines synergistically regulate *Kynurenine Pathway* activity [75], [123]. The pro-inflammatory effects of stress in the CNS is well established [7], [24] and it has been demonstrated that cytokine induction is necessary for Ido1 upregulation by stress [39], [71]. Although some work has investigated the regulation of DOs by stress within the CNS and periphery, the cellular origins responsible for DO induction remain undefined.

Kynurenine itself is not considered a neuroactive metabolite [32], albeit increased DO activity is required for depression-like behaviors following stress [56], [71]. Instead, Kyn is further metabolized down the *Kynurenine Pathway* into other neuroactive metabolites, i.e. kynurenines. This is especially relevant to the CNS, owing to the remarkable cellular specificity in the production of kynurenines [32]. Most notably, astrocytes are enzymatically equipped to produce kynurenic acid (KynA) a glutamate (NMDA) and acetylcholine (α_7nACh) receptor antagonist [42], [46], while microglia produce quinolinic acid (QuinA) and 3-hydroxykynurenine (3-HK) which are NMDA receptor agonists [51], [124]. A recent study found that chronic unpredictable stress increased central Ido1 and Tdo2 mRNA coincident with increased KynA

concentrations, but unchanged 3-HK [125]. This would suggest a specific role for astrocytes in stress-induced DO induction.

Herein we report induction of all three DOs by acute restraint-stress specifically within astrocytes. We have expanded on recent work investigating DO-regulation by stress [119] to include the regulation of recently described alternatively-spliced DO mRNA transcripts [57], [126]. Moreover, since acute stress increases Kyn levels in both plasma and brain [36], [37], we include DO-regulation by stress in liver, the major tryptophan metabolizing organ primarily via Tdo2. Finally, since there is evidence of changes in Ido2 expression within Ido1-knockout ($Ido1^{KO}$) mice [127], [128], we expanded our study to include stress-induced DO-regulation in brain, astrocytes and liver of $Ido1^{KO}$ mice.

2.3 Methods

Animals

C57BL/6J (wild-type) or $Ido1^{KO}$ mice (The Jackson Laboratory, Bar Harbor, ME, USA) were used to establish breeding colonies to supply male mice for experiments. Mice were housed on a reversed 12 h light-dark cycle with ab lib access to food and water. Mice were individually housed at least 1 week prior to experiments. Mice were 14-15 weeks of age at the time of treatment. All animal procedures were approved by the Institutional Animal Care and Use Committee and performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council).

Study design

Restraint-stress

Restraint-stress was initiated at the onset of the dark cycle (10 am) and maintained for 3 h using ventilated syringes [129]. Mice were euthanized 2 h after the cessation of restraint, at which time wild-type mice had a significant reduction in body weight associated with restraint-stress (control change in body weight 0.1 ± 0.1 g vs. restrained -0.9 ± 0.1 g, $p<0.001$). A similar response was seen with $Ido1^{KO}$ mice (control 0.4 ± 0.1 g vs. restrained -0.7 ± 0.2 g, $p<0.001$).

Following euthanasia, caval blood was collected into heparinized syringes. Samples were centrifuged and plasma collected. Mice were intracardially perfused using cold PBS plus 2 mM EDTA. After perfusion, samples were collected from separate cohorts of mice for either 1) whole brain sampling followed by glia enrichment or 2) brain region and liver collection as described in sections 2.2.2 and 2.2.3, respectively.

Brain sampling and Glia enrichment: mouse cohort 1

Brains were removed and placed in ice-cold Hank's balanced salt solution then homogenized with a GentleMACS dissociator using Neural Tissue Dissociation kits (130-093-231, Miltenyi Biotec Inc.) per manufacturer instructions. Immediately after homogenization, an aliquot of 'brain' was removed and stored in TRIzol (15596018, Ambion by Life Technologies) for later RNA isolation. The 'brain' samples were collected from homogenates before removal of astrocytes and microglia and thus represent expression by all cells and all brain regions in their original proportion. Remaining homogenate was demyelinated by centrifugation in 20% **i**sotonic Percoll (E0414-1L, Sigma-Aldrich, St. Louis, MO). The cell pellet was re-suspended then partitioned into two fractions for positive-selection using either anti-Cd11b (130-093-634) or

anti-Glast labeling microbeads (130-095-826). Microglia ($Cd11b^+$) and astrocytes ($Glast^+$) were enriched using MACS MS magnetic separation columns. Microglia and astrocyte populations were suspended in TRIzol and stored at -80°C for later RNA isolation [130]. Glia enrichment was verified by comparing Glast1 and Cd11b mRNA expression to the 'brain' homogenate from which they were derived. Glast1 expression was greater in enriched astrocytes compared to brain in wild-type mice (5.9 ± 0.4-fold, $p<0.001$) and $Ido1^{KO}$ mice (12.0 ± 1.0-fold, $p<0.001$). Cd11b expression was greater in microglia preparations compared to brain from wild-type mice (265.6 ± 10.8-fold, $p<0.001$).

Brain region and liver collection: mouse cohort 2

Brain-regions (prefrontal cortex (PFC), striatum (Stri), hippocampus (Hippo), hypothalamus (Hypo)) and livers were collected and frozen for later RNA extraction. The remaining brain (sans PFC, Stri, Hippo and Hypo) was frozen for analysis of Kyn.

Gene Expression by qPCR

RNA was extracted from brain, astrocytes, microglia, PFC, Stri, Hippo, Hypo and liver, then reverse-transcribed (4368813, Applied Biosystems). Resulting cDNA was used for quantitative polymerase chain reaction (qPCR) using TaqMan Universal PCR Master Mix (4324020, Applied Biosystems). Expression of each test gene were normalized to the reference gene (Gapdh) using the $2^{-\Delta\Delta Ct}$ method [131]. Gene expression in naïve control (Ctrl) brain or Ctrl PFC is set to 1.0, with samples from the same experiments expressed relative to appropriate controls. Gene structure and mRNA transcripts for the DOs are shown in Figure 3. Assays designed to quantify the various DO transcripts are described in Table 1. PCR was performed with probe-based assays (IDT, Coralville, Iowa). Custom assays were designed using the IDT PrimerQuest® Design Tool. Assays for Kat2 (Mm.PT.49.16077568), Kmo (Mm.PT.56.31570400),

Kynu (Mm.PT.56.12643855) and Haao (Mm.PT.58.29327685) were predesigned by IDT. Because of low expression, some transcripts are 'not detected' (i.e. C_t values 'undetermined') thus preventing calculation of relative gene expression following induction; for analysis a C_t value of 40.0 is assigned when this occurs.

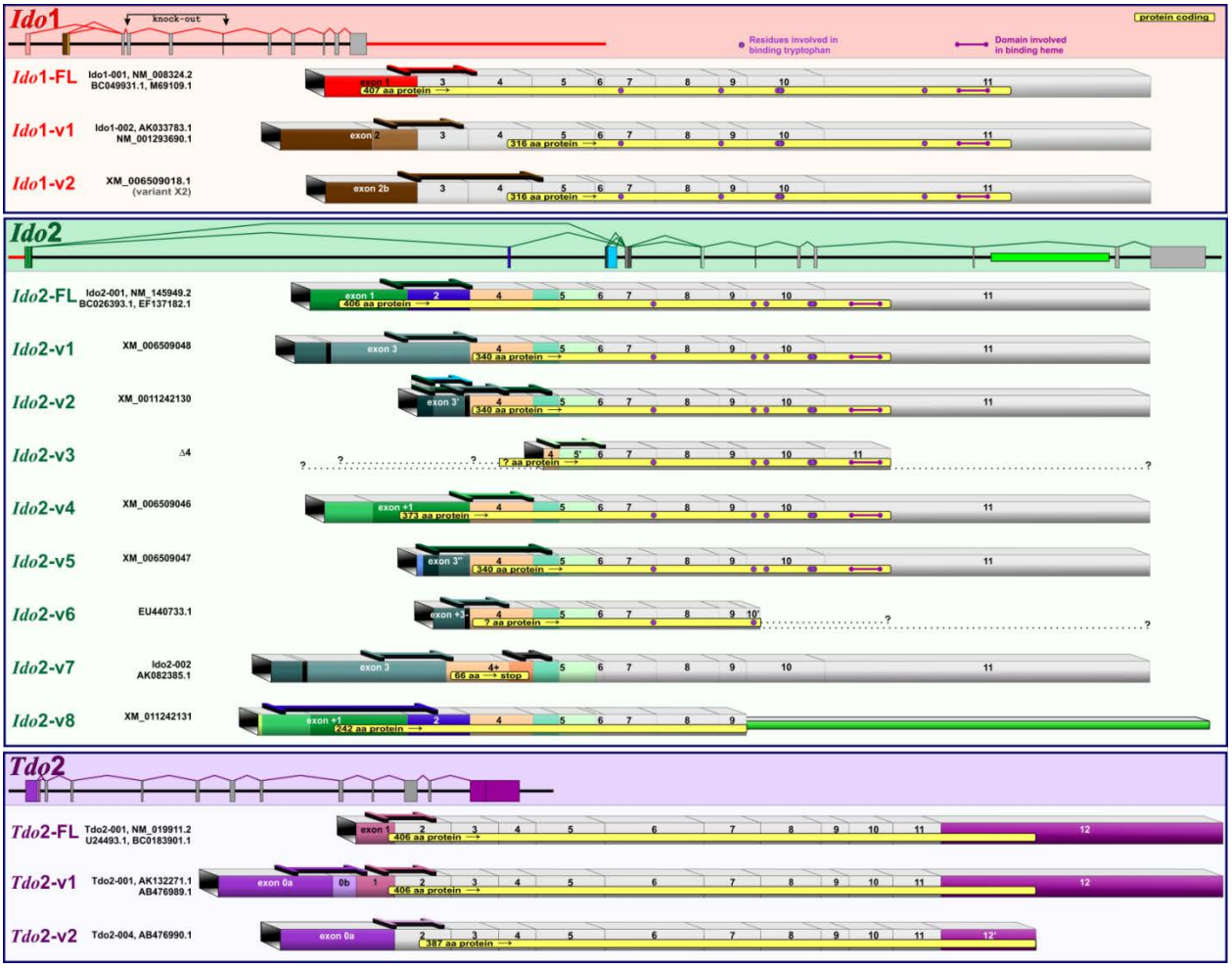

Figure 3. Gene structure and transcripts for murine Ido1, Ido2 and Tdo2. Like human DOs, murine DO gene-processing results in the expression of multiple mRNA transcripts. Our previous (Brooks et al., 2016a, 2016b) and current work clearly show distinct transcripts are utilized to fine-tune regulation of the Kynurenine Pathway.

Table 1. Assay specifics for analysis of murine Ido1, Ido2 and Tdo2 steady-state gene expression.

Transcript	Exon Span	Key	Catalog # or assay name		Primer/Probe Sequence	Amplicon, bp
Ido1-FL	1-3-4		Mm.PT.42.8645095	Forward	TTTGCTCTACCACATCCACTG	117
				Probe	6-FAM CAGATTTCT Zen AGCCACAAGGACCCAGG IABkFQ	
				Reverse	GCAGCTTTTCAACTTCTTCTCG	
Ido1-v1	2-3		Ido1-v ex 2-3	Forward	GACCCCGGACGGTAAAATTAT	138
				Probe	6-FAM TCGGGCAGC Zen TCCACATTACAATTCA IABkFQ	
				Reverse	TCTCAATCAGCACAGGCAG	
Ido1-v2	2b-3-4-5		Ido1-X2-mouse Set 1	Forward	CGGACGGTGGAGCTG	256
				Probe	6-FAM ATTGAGAAC Zen GGGCAGCTTCGAGAA IABkFQ	
				Reverse	CGCAGTAGGGAACAGCAATA	
Ido2-FL	1-2-4		Mm.PT.42.8856312	Forward	GGAGATACCACATTTCTGAGGA	114
				Probe	6-FAM CCAGAGGAT Zen TTGGAAGGAGAAAGCCAT IABkFQ	
				Reverse	CGATTAAGTGAGGAAGTCTGAGG	
Ido2-v1	3-4		Ido2-v ex 3-4	Forward	GGACTTTACATCCCTAACCTCAC	131
				Probe	6-FAM CCTCAGCTT Zen CTCGAACCCTGTAACTGTA IABkFQ	
				Reverse	CTGCTCACGGTAACTCTTTAGG	
Ido2-v2	3'-4		Ido2-X4 Set 4	Forward	AAGCCTGCGGAGCAAAG	88
			Ido2-X2-mouse Set 1	Probe	6-FAM TGAAGAGAT Zen GAGCAATGAGCCGGT IABkFQ	
			Ido2-v2-ex3-4 set2	Reverse	GAGGCATCTGTCCTGCCT	
Ido2-v3	4-5'-6-7		Ido2-v4-Mz Set1	Forward	CCCCAAAAGGTATCCAGGAACT	107
				Probe	6-FAM CTGACCTGG Zen TGCTGACAAACTGGA IABkFQ	
				Reverse	ACTGATTTCCAACGGTCCTTCT	
Ido2-v4	+1-4-5		Ido2-X1b-mouse Set 2	Forward	CCAAATCCTCTGATGCCTCTC	148
				Probe	6-FAM TAAAGAGTT Zen ACCGTGAGCAGCGCC IABkFQ	
				Reverse	AAAGGTGCTGCCAAGATCTC	
Ido2-v5	3''-5		Ido2-X2-mouse Set 1	Forward	AAGCCTGCGGAGCAAAG	244/253
				Probe	6-FAM TGAAGAGAT Zen GAGCAATGAGCCGGT IABkFQ	
				Reverse	CCAAGTTCCTGGATACCTCAAC	
Ido2-v6	+3'-4		Ido2-v2-ex3-4 set2	Forward	GAGCTGAAGAGATGAGCAATGA	85/85
				Probe	6-FAM AGGACAGAT Zen GCCTCTCCTGGACT IABkFQ	
				Reverse	ACGGTAACTCTTTAGGAATCTGC	
Ido2-v7	4+-5		Ido2-v1-ex4'-5 set1	Forward	AGGTTCCGTTGCCTAGTTTC	101
				Probe	6-FAM AAGAGGGTG Zen CTGCCAAGATCTCTT IABkFQ	
				Reverse	CCAAGTTCCTGGATACCTCAAC	
Ido2-v8	+1-2		Ido2-X5 Set 1	Forward	CCTCAGGGCAAGGTTCTC	294
				Probe	6-FAM ACCGCACAA Zen GTACAACCACACAGA IABkFQ	
				Reverse	CTGGCGGTTCTCGATTAAGT	
Tdo2-FL	1-2		Mm.PT.42.9084201.g	Forward	CCTGAGACACTTCAGTACTATGAG	99/99
				Probe	6-FAM CCCGTTTGC Zen AGGAAACAGTGTAGGA IABkFQ	
				Reverse	CTGTCTTCTTCATTGTCCTCCA	
Tdo2-v1	0a-0b-1		Tdo2 variant1 exons 0a 0b-1.2	Forward	CCAGTACGAAATGAGATCCGG	132
	1-2		Mm.PT.42.9084201.g	Probe	6-FAM AGACACAGC Zen CAATCAGCACCCA IABkFQ	
				Reverse	AGGTTTGCTAGGTCAGGAATG	
Tdo2-v2	0a-2		Tdo2 variant2 exons 0a 1.2	Forward	GAAATGAGATCCGGGCTAAGAG	78
				Probe	6-FAM TGGGTGCTG Zen ATTGGCTGTGTCT IABkFQ	
				Reverse	GTGTATCTTTTATGTATCCTGATTGCC	

Ido2-v5 and Ido2-v6 sequence lie within Ido2/v2 (thus it is not possible to design an assay specific to Ido2-v5 or Ido2-v6 that does not also quantify Ido2-v2). We have data indicating distinct differences in the regulation of these three transcripts [126]. Tdo2-FL's complete sequence lies within Tdo2-v1 (thus it is not possible to design an assay specific to Tdo2-FL that does not also quantify Tdo2-v1). Our current and published [57], [126] data show distinct differences in the expression and regulation of Tdo2-FL and Tdo2-v1, indicating that they are distinctly expressed transcripts. Names for Ido2-v3:Δ4, Tdo2-FL/-v1/-v2 and Tdo2 exon 0a and 0b exon designations are shown in an attempt to agree with published nomenclature [55], [100]. Specifics shown for each qPCR assay include transcript location, catalog numbers (Mm.PT…) or our custom assay names, primer/probe sequences and confirmed amplicon sizes.

Kynurenine levels by high performance liquid chromatography (HPLC)

Brain samples were suspended in ice cold buffer (0.1 N perchloric acid + 25 µM ascorbic acid) at 200 mg wet weight/ml and disrupted by sonication. Samples were incubated for 30 min then centrifuged at 12,000 *g* for 5 min at 4°C. Supernatants were collected and loaded into Spin-X filters (Corning, NY, USA) and centrifuged at 10,000 *g* for 5 min at 4°C. Filtrates were used for HPLC analysis. Plasma samples were processed for analysis by HPLC as previously reported [132]. HPLC mobile phase consisted of 75 mM monosodium phosphate (pH 4.6), 25 µM EDTA and 0.01% triethylamine prepared in either 4.5 or 6% acetonitrile for brain and plasma extracts, respectively. Chromatogram peaks were integrated using EZChrom SI software (Agilent Technologies, Santa Clara, CA, USA). Kyn standards were made to encompass levels in the samples.

Statistics

Two-way ANOVA was used to compare the relative expression of genes between astrocytes, microglia and brain, and to compare brain-regions and liver, while T-tests were used to determine the effects of stress. Analysis was performed using SigmaPlot 14.0 software. Significance was set at $p \leq 0.05$.

2.4 Results

Acute stress increases gene expression of markers for HPA axis activation and inflammation

FK506-binding protein 51 (Fkbp51) expression was increased by restraint-stress in both brain and liver (Figure 4A). These data confirm a HPA axis response [133], [134]. Some effects of restraint-stress are mediated by increases in pro-inflammatory cytokines. Restraint-stress

increased IL-1β (Figure 4B) and TNFα (Figure 4C) expression within brain, confirming an inflammatory response. Although relative expression of IL-1β and TNFα was higher in liver vs. brain, a stress-induced inflammatory response was not observed in liver. Also, IFNγ gene expression was unaffected by restraint-stress in brain and liver. IFNγ protein concentrations in plasma from naïve and restrained mice were below detection-limits (data not shown).

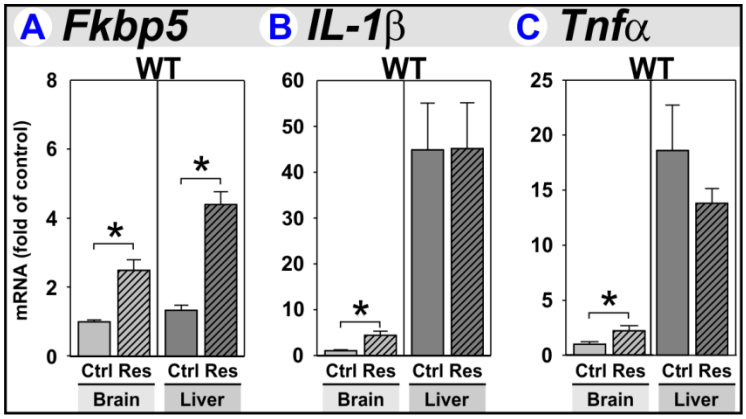

Figure 4. Acute stress increases gene expression of markers for stress and inflammation. Perfused brain and liver tissue obtained from wild-type (WT) control (Ctrl) and restraint-stressed (Res) mice sacrificed 2 h after a 3 h restraint were used to assess **(A)** HPA activation by quantifying Fkbp51 expression and an inflammatory response by quantifying **(B)** IL-1β and **(C)** TNFα expression. *p<0.05 Ctrl vs. Res.

Acute stress increases plasma and brain kynurenine in wild-type, but not Ido1^{KO} mice

Plasma concentrations of Kyn were increased by restraint-stress in wild-type, but not Ido1^{KO} mice (Figure 5A). Brain concentrations of Kyn were also increased by stress in wild-type, but not Ido1^{KO} mice (Figure 5B). Since the DOs are rate-limiting enzymes in the metabolism of Trp to Kyn [32], these data verify restraint-stress activation of the *Kynurenine Pathway* and the necessity of Ido1 for increased Kyn.

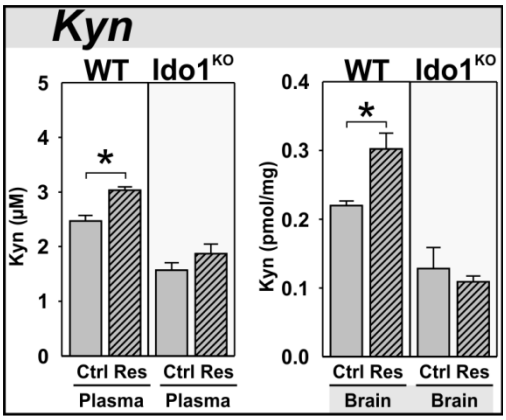

Figure 5. Acute stress increases plasma and brain kynurenine in wild-type, but not Ido1^{KO} mice. Two hours after a three hour restraint-stress **(A)** plasma and **(B)** brain from WT and Ido1^{KO} mice were analyzed for kynurenine (Kyn) by HPLC. *p<0.05 comparing control (Ctrl) to restraint-stress (Res) samples.

Acute stress increases Ido1-v1 expression in astrocytes and select brain-regions

Ido1 expression in the mouse brain is reported to both increase and remain unchanged following acute stress. Based on our nomenclature (Figure 3), these studies used PCR assays amplifying either Ido1-FL [39], [120] or all Ido1 transcripts simultaneously, Ido1-Tot [56]. We independently quantified expression three Ido1 transcripts to refine this issue. In agreement with our previous work [57], Ido1-FL and Ido1-v2 transcripts were poorly expressed in brains and livers of naïve mice. Their expression was not induced by restraint-stress (data not shown).

Ido1-v1 is well expressed in the mouse brain and numerically, but non-significantly, elevated by restraint-stress (Figure 6). Initially, this suggests that Ido1-v1 expression is not altered by restraint-stress; however, a more in-depth investigation reveals the nature of Ido1-v1 expression. Ido1-v1 expression is induced 4.8-fold in astrocytes, but not in microglia, isolated from stressed mice. The stress effect is not only cell-type specific but brain-region specific, disguising significant effects when whole-brain is assessed. Ido1-v1 expression was highest in striatum followed by hypothalamus relative to other brain-regions. Restraint-stress increased Ido1-v1 in prefrontal cortex and hypothalamus. As expected, Ido1^{KO} mice did not express Ido1-

v1 (Figure 6), Ido1-FL or Ido1-v2 (not shown). Hepatic expression of Ido1-FL, Ido1-v1 and Ido1-v2 was low and not altered by stress. Thus, Ido1 expression exhibits brain-region, glial and transcript-specificity within naïve and restrained wild-type mice.

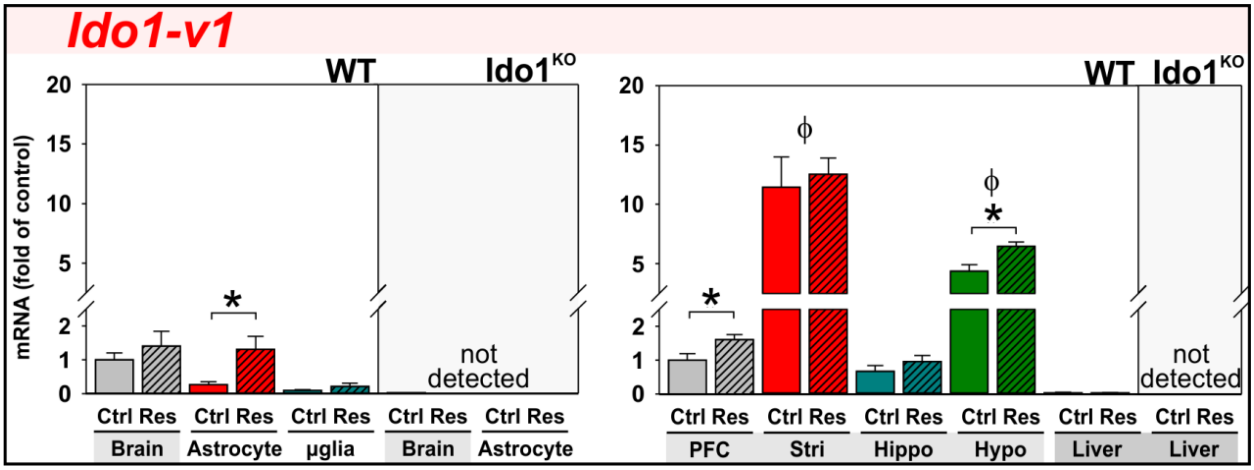

Figure 6. Acute stress increases Ido1-v1 expression in astrocytes and select brain-regions. Tissue and glia from control (Ctrl) and restraint-stressed (Res) mice sacrificed 2 h after a 3 h restraint were analyzed for Ido1-v1 expression. Expression was quantified in whole-brain, glia, liver and several brain-regions: the prefrontal cortex (PFC), striatum (Stri), hippocampus (Hippo) and hypothalamus (Hypo). Samples from $Ido1^{KO}$ mice did not express any Ido1 transcript. Ido1-FL and Ido1-v2 transcripts were not detected by qPCR. *$p<0.05$ comparing Ctrl to Res within tissue or glia. $^{\phi}p<0.05$ compared to other brain-regions.

Acute stress increases Ido2-v3 expression in astrocytes

To our knowledge, there is only one report examining the effect of stress on Ido2 expression. Using an assay that detects all transcripts (Ido2-Tot), forced-swimming-associated stress increased hippocampal Ido2 expression in BALB/c, but not C57BL/6J, mice [120]. We quantified expression of Ido2 (Figure 3) for transcript-, tissue- and glial-specific responses between naïve and restrained mice. Of eight Ido2 mRNA variants, restraint-stress remarkably increased the expression of only one Ido2 transcript; this transcript-specificity would make it difficult to detect changes when simultaneously quantifying all transcripts, i.e. Ido2-Tot.

Ido2-FL is poorly expressed in the naïve mouse brain except within the striatum where Ido2-FL expression is 10-fold higher (Figure 7A) than other brain-regions. In striking contrast, hepatic Ido2-FL is markedly higher in relation to brain-regions. While restraint-stress had no effect on central Ido2-FL, hepatic Ido2-FL expression in wild-type and $Ido1^{KO}$ mice was decreased following restraint.

Ido2-v3 is well expressed in the mouse brain, but stress-induced changes are not realized when analyzing whole-brain or brain-regions (Figure 7B). The low basal expression in astrocytes and microglia relative to brain indicates that most of the Ido2-v3 in naïve brain is expressed by stress-insensitive cells. Nonetheless, Ido2-v3 expression is increased by stress 13-fold in astrocytes. In $Ido1^{KO}$ mice, Ido2-v3 expression by astrocytes is not increased by stress. In wild-type mice, hepatic expression of Ido2-v3 is greater relative to brain-regions; and hepatic Ido2-v3 is decreased by stress, albeit significant only for $Ido1^{KO}$ mice.

Ido2-v4 is also poorly expressed in the naïve mouse brain except within the striatum (Figure 7C) where Ido2-v4 expression is highest [57]. Again, hepatic expression of Ido2-v4 is markedly higher in relation to brain-regions. While not regulated by stress within the brain, stress decreased hepatic Ido2-v4 expression in wild-type and $Ido1^{KO}$ mice.

Thus, expression of Ido2-FL, Ido2-v3 and Ido2-v4 are stress sensitive, lowered in liver and Ido2-v3 is elevated in astrocytes. Other Ido2 transcripts exhibit unique patterns of expression across tissues and brain-regions, but their expression is relatively independent of stress (Figure 8).

Compared to wild-type mice, the brains of $Ido1^{KO}$ mice are strikingly deficient in several Ido2 transcripts, including 78% reduction in Ido2-v3 (Figure 7B), 84% reduction in Ido2-v1, 92%

reduction in Ido2-v2, 22% reduction in Ido2-v5, 57% reduction in Ido2-v6 and loss of detectable Ido2-v7 (Figure 8). This loss of Ido2 in Ido1^{KO} is cell- and tissue-specific, and was previously reported to occur in B-lymphocytes, but not liver [100]. We confirm their finding showing that relative expression of various Ido2 transcripts is similar in the liver between wild-type and Ido1^{KO} mice (Figure 7 and Figure 8). The decrease in Ido2-v3 within brains of Ido1^{KO} mice is not mediated astrocytes, as Ido2-v3 in astrocytes from Ido1^{KO} mice is numerically greater than astrocytes from wild-type mice. Thus, loss of Ido2-v3 occurs in another cell-type within the brain. Additionally, although Ido2-v3 is induced by stress in astrocytes from wild-type mice, it was not increased in Ido1^{KO} mice. Thus, the regulation of Ido2 is transcript-, tissue- and cell-type-specific in wild-type mice, and genetic deletion of Ido1 perturbs central Ido2 expression. Importantly, when interpreting physiologic differences between wild-type and Ido1^{KO} mice, diminished Ido2 expression in the brain should be considered.

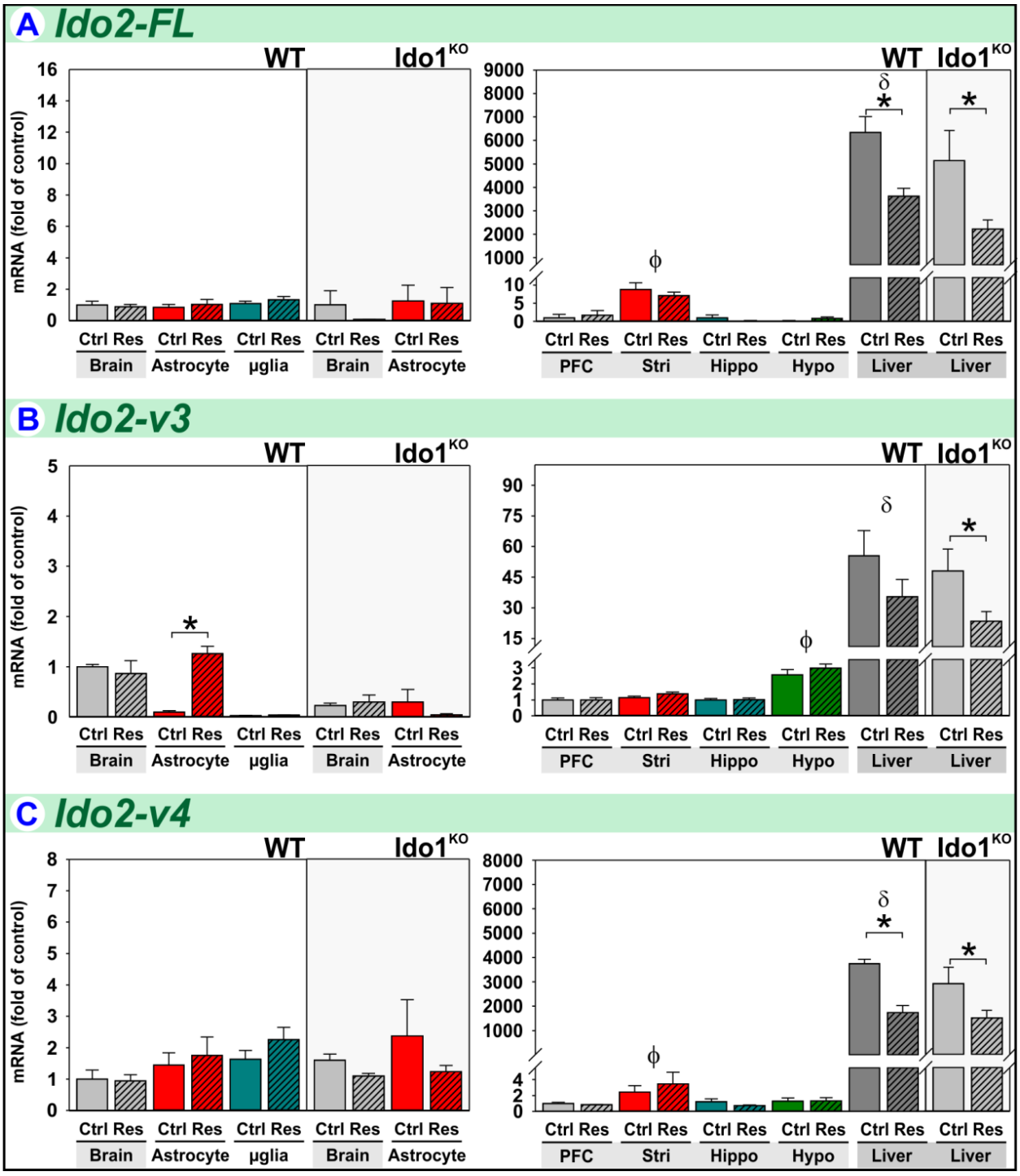

Figure 7. Acute stress increases Ido2-v3 expression in astrocytes. Tissue and glia from control (Ctrl) and restraint-stressed (Res) mice sacrificed 2 h after a 3 h restraint were analyzed for **(A)** Ido2-FL, **(B)** Ido2-v3 and **(C)** Ido2-v4 expression. *p<0.05 comparing Ctrl to Res within tissue or glia. $^{\phi}$p<0.05 compared to other brain-regions. $^{\delta}$p<0.05 liver compared to all brain-regions.

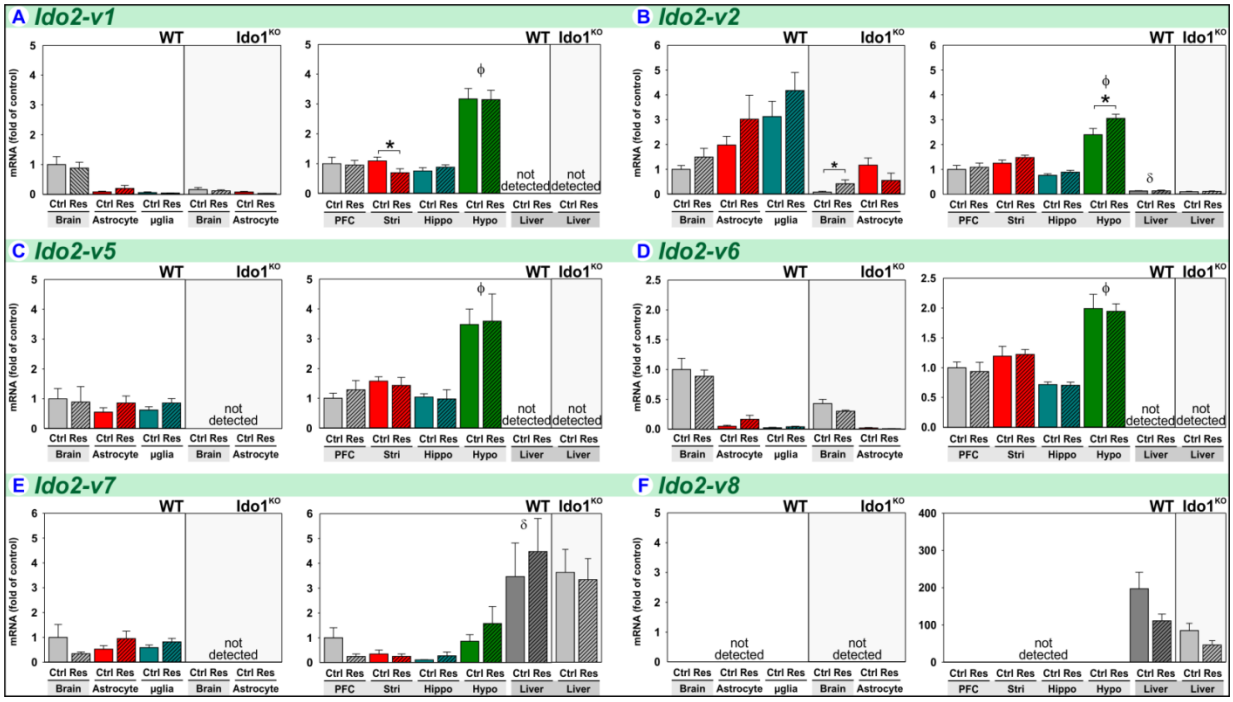

Figure 8. Additional Ido2 transcripts and their expression following acute stress. Tissue and glia from control (Ctrl) and restraint-stressed (Res) mice sacrificed 2 h after a 3 h restraint were analyzed for additional Ido2 transcripts. **(A)** Ido2-v1 expression in brain is greater than astrocyte and microglia levels, indicating these cell-types are not the major source of Ido2-v1. Hypothalamic Ido2-v1 expression was greater than all other brain-regions. Stress slightly decreased Ido2-v1 relative to controls in the striatum. Ido2-v1 expression was not detectable in the liver. **(B)** Ido2-v2 expression was greater in microglia than brain with brain and astrocytes having similar expression levels, indicating that microglia and astrocytes are a major source of central Ido2-v2. In Ido1^{KO} mice, Ido2-v2 was greater in astrocytes compared to brain, and increased following restraint-stress relative to Ctrl. Within brain-regions, Ido2-v2 expression differed with Hypo > PFC = Stri > Hippo > liver. Hypo Ido2-v2 was increased ~28% by stress relative to Ctrl. **(C)** Ido2-v5/v2 expression did not differ in brain, astrocytes or microglia of Ctrl or stressed mice, indicating a uniform expression pattern for this transcript. Ido2-v5/v2 expression in the hypothalamus was greater than all other brain-regions. Hepatic Ido2-v5/v2 expression was not detected. Ido2-v5/v2 expression was not detected in brain or astrocytes of Ido1^{KO} mice. **(D)** Brain Ido2-v6/v2 expression was greater than astrocyte and microglia, indicating these cell-types are not the major source of brain Ido2-v6/v2. In Ido1^{KO} mice, brain Ido2-v6/v2 expression was also greater than astrocyte levels. Ido2-v6/v2 expression was greater in Hypo than all other brain-regions. Hepatic Ido2-v6/v2 was not detectable. **(E)** Ido2-v7 expression did not differ in brain, astrocytes or microglia of Ctrl or stressed mice or across brain-regions, indicating a uniform expression pattern for this transcript. Ido2-v7 was not detected in Ido1^{KO} mice brain or astrocytes. Hepatic Ido2-v7 expression levels were greater than all other brain-regions. **(F)** Ido2-v8 expression was only detected in liver. *p<0.05 comparing Ctrl to Res samples within tissue or glia. $^{\phi}$p<0.05 compared to other brain-regions. $^{\delta}$p<0.05 liver compared to all brain-regions.

Overall these additional Ido2 transcripts are relatively impervious to stress. However, Ido2 transcripts fall into two general categories: 1) hepatic expression > than brain (Ido2-FL, Ido2-v3, Ido2-v4 (Figure 7), Ido2-v6 and Ido2-v8 (Figure 8)); 2) brain expression > than liver (Ido2-v1, Ido2-v2, Ido2-v5 and Ido2-v7 (Figure 8)). This illustrates a distinct regulatory mechanism driving the expression of Ido2, albeit with as yet unrecognized functional consequences.

Acute stress increases Tdo2-FL expression in brain, astrocytes and liver

Tdo2 was the first DO investigated within the context of acute stress, with early reports describing increases in hepatic Tdo2 activity [117], [135], [136]. However, Tdo2 expression in the frontal cortex of the rat brain was unchanged after acute stress despite an increase of Tdo2 in liver using a qPCR assay that quantified all Tdo2 transcripts, i.e. Tdo2-Tot [56]. The current report is the first to quantify the regulation of the three known Tdo2 transcripts (Figure 3) in stressed mice to identify distinct regulatory profiles.

Tdo2-FL expression is increased to 2.3-fold of controls in brain by restraint-stress (Figure 9A). This effect is paralleled by a 1.8-fold increase within astrocytes. Tdo2-FL expression in microglia is lower than brain and astrocytes and unaffected by stress. Tdo2-FL expression within astrocytes from $Ido1^{KO}$ mice was also doubled by restraint-stress, but not within the brains from which they were derived. Astrocyte Tdo2-FL expression is greater in $Ido1^{KO}$ mice compared to the brain of wild-type mice, suggesting genetic deletion of Ido1 results in specific compensatory Tdo2-FL upregulation within astrocytes. Although stress increased Tdo2-FL expression in brain from wild-type mice, stress did not increase Tdo2-FL in brain of $Ido1^{KO}$ mice. This finding suggests another brain cell-type, possibly neurons [54], expressing Tdo2-FL is only stress-sensitive when Ido1 is intact. Tdo2-FL is considerably higher in the liver relative to brain-regions and increased by stress in both wild-type and $Ido1^{KO}$ mice.

Tdo2-v1 is increased to 10.1-fold of controls by restraint-stress in brains from wild-type mice (Figure 9B). Although Tdo2-v1 expression is greater in astrocytes and microglia relative to whole-brain, its expression in these cells is unaffected by restraint. These data suggest the stress-induced increase in brain Tdo2-v1 is mediated by neurons (or other glia) which also express Tdo2 [54]. Unlike Tdo2-FL, Tdo2-v1 expression is similar across brain-regions. Thus,

these data illustrate a unique expression pattern vs. Tdo2-FL (despite the entire Tdo2-FL sequence encompassed within Tdo2-v1, Figure 3). Like Tdo2-FL, there is no increase in brain Tdo2-v1 expression in $Ido1^{KO}$ mice, indicating a requirement of Ido1 for brain Tdo2-v1 induction. In wild-type mice, hepatic Tdo2-v1 expression is greater than in brain-regions, but not significantly affected by restraint-stress.

Tdo2-v2 expression in brain of wild-type mice is increased 7.3-fold by restraint-stress (Figure 9C). Tdo2-v2 expression in both astrocytes and microglia is greater than brain levels, yet unaffected by restraint. Like Tdo2-v1, these data suggest the stress-induced increase in brain Tdo2-v2 is mediated by neurons or other glia. Tdo2-v2 expression in astrocytes from $Ido1^{KO}$ mice is decreased slightly but significantly by restraint. Tdo2-v2 expression is similar across brain-regions. In wild-type mice, Tdo2-v2 levels in liver were greater than brain-regions and increased by restraint.

Therefore, all three Tdo2 transcripts are increased by restraint-stress in brains of wild-type mice. The increase in brain Tdo2-FL parallels the increase in astrocytes, whereas the increases in Tdo2-v1 and Tdo2-v2 are mediated by unidentified cell-types. Only Tdo2-FL and Tdo2-v2 are increased by restraint-stress in liver relative to control mice. Thus, there is transcript-, tissue- and cellular-specificity of Tdo2 expression and regulation by restraint-stress.

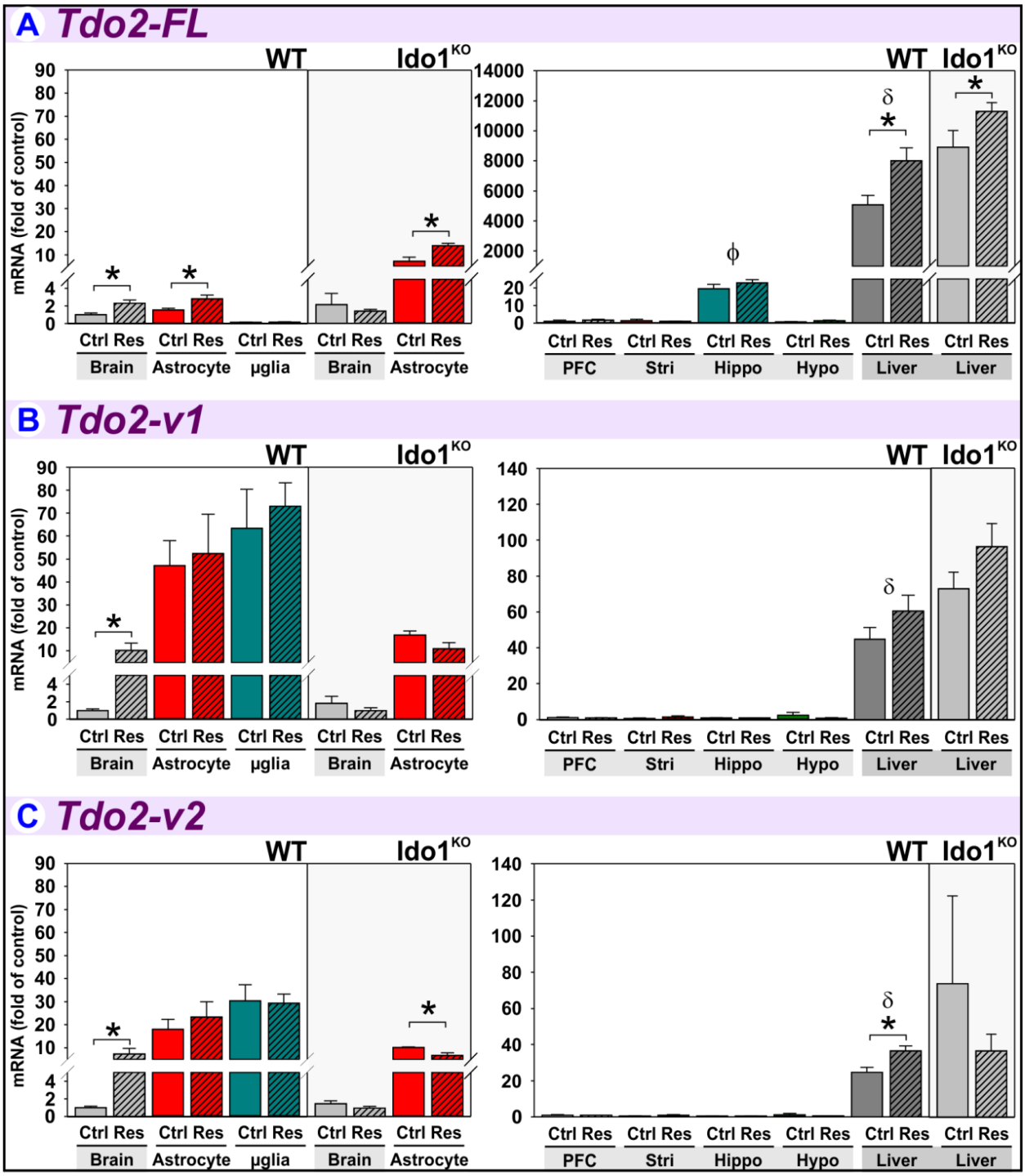

Figure 9. Acute stress increases Tdo2-FL expression in liver, brain and astrocytes. Tissue and glia from control (Ctrl) and restraint-stressed (Res) mice sacrificed 2 h after a 3 h restraint were analyzed for **(A)** Tdo2-FL/v1, **(B)** Tdo2-v1 and **(C)** Tdo2-v2 expression. *p<0.05 comparing Ctrl to Res within tissue or glia. $^{\phi}$p<0.05 compared to other brain-regions. $^{\delta}$p<0.05 liver compared to all brain-regions.

Other Kynurenine Pathway related gene expression & regulation by stress

The DOs are rate-limiting for Trp to Kyn metabolism, but Kyn itself is further metabolized into neuro- and immune-active kynurenines in a cell-specific manner. This specificity is achieved by differential expression of enzymatic machinery downstream of the DOs [29]. Thus, we expanded our analysis to include enzymes further along the *Kynurenine Pathway*.

Kat2 (kynurenine-2-oxoglutarate aminotransferase) converts Kyn to KynA. Kat2 expression is enriched in astrocytes compared to microglia and highly expressed in liver. Within brain regions, Kat2 expression is highest in the hypothalamus; however, Kat2 expression is unaffected by stress (Figure 10A). These data confirm previous reports of astrocyte enrichment [47], [137], [138].

Kynu (kynureninase) initiates Kyn metabolism to QuinA. Kynu expression is greater in microglia relative to astrocytes and considerably higher in liver (Figure 10B). These data confirming previous reports of microglial enrichment of this enzyme within the brain [42], [139]. Kynu expression is highest in the hypothalamus with expression largely unaffected by stress, the exception being a decrease in the hippocampus.

Kmo (kynurenine 3-monooxygenase) also initiates Kyn metabolism to QuinA. Kmo expression is greater in microglia relative to astrocytes, but again considerably higher in liver. These data confirm previous reports of microglial enrichment of this enzyme in the brain [42], [139]. Kmo expression is increased ~50% in astrocytes and ~20% in microglia by stress (Figure 10C).

Haao (3-hydroxyanthranilate 3,4-dioxygenase) acts downstream of Kynu and Kmo to complete QuinA synthesis. Haao expression is greater in microglia relative to astrocytes, but again considerably higher in liver. Haao expression is slightly but significantly decreased by stress in both microglia and liver (Figure 10D). Again, these data confirm preferential expression of Haao by microglia in the brain [42], [51].

The extremely high levels of Tdo2, Ido2 and especially the downstream enzymes within the liver (relative to brain), attest to its ability to efficiently generate niacin and NAD [140]. Overall, since the DOs are considered rate-limiting in the *Kynurenine Pathway,* these changes in downstream enzymes probably do not result in an overall shift in the relative ability to produce downstream kynurenines; that is to say astrocytes will generate primarily KynA and microglia QuinA as limited by Kyn production by the DOs.

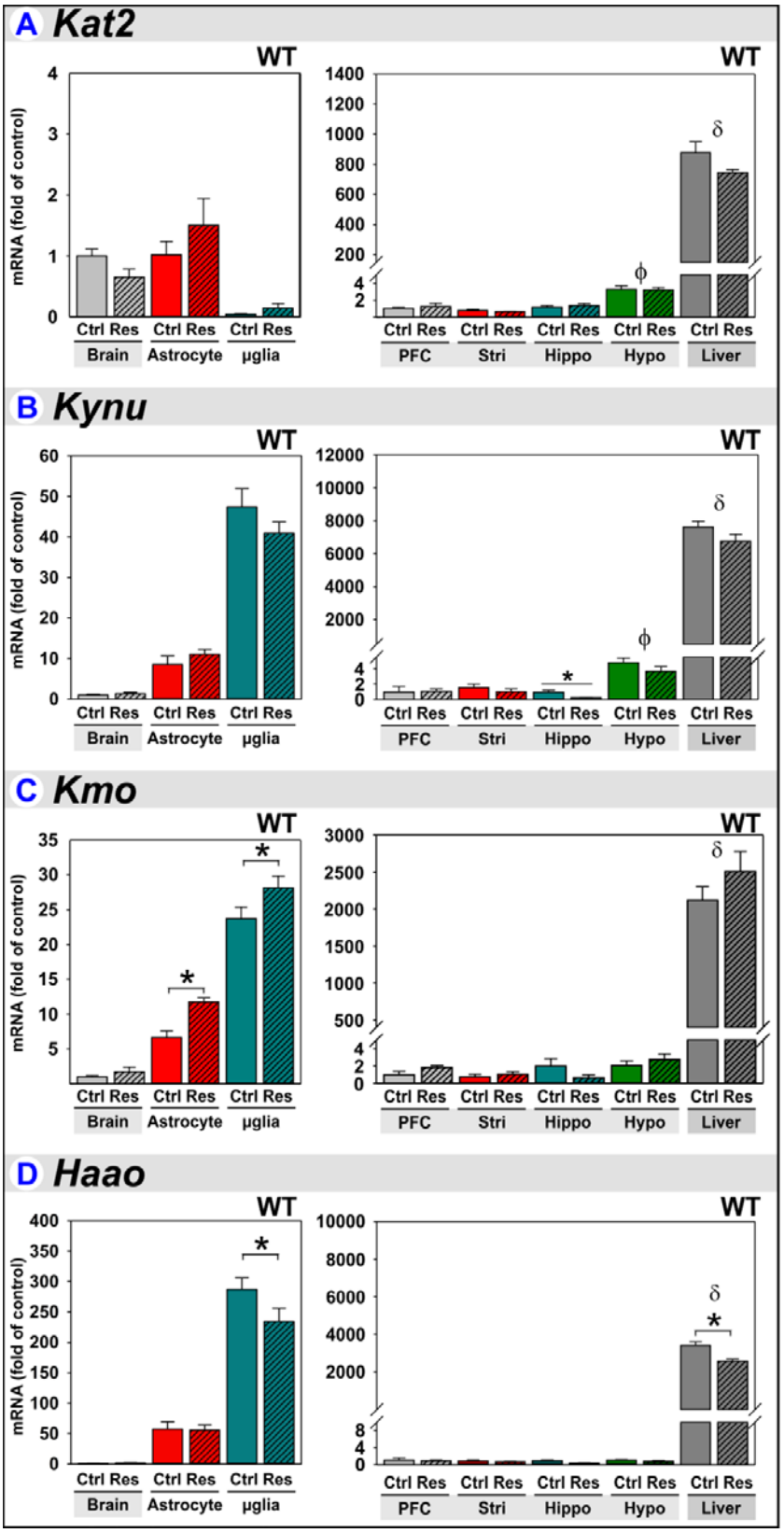

Figure 10. Other Kynurenine Pathway related gene expression & regulation by stress. Tissue and glia from control (Ctrl) and restraint-stressed (Res) mice sacrificed 2 h after a 3 h restraint were analyzed for **(A)** Kat2, **(B)** Kynu, **(C)** Kmo and **(D)** Haao expression. *p<0.05 comparing Ctrl to Res within tissue or glia. $^{\phi}$p<0.05 compared to other brain-regions. $^{\delta}$p<0.05 liver compared to all brain-regions.

2.5 Discussion

Acute restraint-stress increased the expression of all three DOs within the mouse brain in a transcript-, tissue- and cell-type specific manner. Brain and plasma levels of Kyn were increased in stressed mice relative to controls demonstrating a functional increase in DO activity following acute restraint-stress. Remarkably, Ido1-v1, Ido2-v3 and Tdo-FL expression was increased by stress in astrocytes, but not microglia. However, in comparison to wild-type mice, we found aberrant Ido2 and Tdo2 expression in $Ido1^{KO}$ mice demonstrating a necessity of Ido1 for normal Ido2 and Tdo2 regulation within the brain. The expression patterns of downstream *Kynurenine Pathway* enzymes support the hypothesis that astrocytes are central producers of KynA while microglia are equipped to produce primarily QuinA. Since only astrocyte DO expression was increased by stress, these finding further implicate a key role for Kyn and KynA within the neurobiology of stress-induced behaviors.

Acute stress increases gene expression of markers for stress and inflammation

The induction of Fkbp51 by restraint-stress in brain and liver (Figure 4A) confirm glucocorticoid receptor activation [133], [134]. However, restraint-stress increased brain, but not liver, expression of pro-inflammatory cytokines, Tnfα and IL-1β (Figure 4B-C), confirming previous reports of increases in brain IL-1β [20], [122] and circulating TNFα and IL-1β following stress, without coincident changes in the liver [38]. While both the HPA axis and autonomic nervous system contribute to the induction of peripheral and central pro-inflammatory cytokines following stress [7], [24], the pro-inflammatory cytokines TNFα [26] and IL-1β [25] also increase glucocorticoid release. Extending our understanding by which inflammatory mediators are regulated by the stress response (or vice versa) is out of the scope of this study;

however, we are interested in how these confirmed stress responses affect the *Kynurenine Pathway*.

Acute stress increases plasma and brain kynurenine in wild-type, but not Ido1^{KO} mice

Following acute stress, increases in central and peripheral Kyn concentrations have been independently verified [36]–[39], [115], [141]. Our data reaffirm acute-stress increases both brain and plasma Kyn and further demonstrates the necessity of Ido1 for restraint-stress-induced increase in Kyn (Figure 5A-B). Although Ido1 is intimately involved in elevated Kyn levels associated with inflammation [33], Ido2 and Tdo2 also have this capability [54]. Thus, the inability of stress to induce Ido2-v3 and Tdo2 variants in Ido1^{KO} mice may also be involved in the lack of Kyn induction in Ido1^{KO} mice.

Acute stress increases Ido1-v1 expression in astrocytes and select brain-regions

Ido1 expression is increased by inflammatory mediators [32], [57], [76] and synergistically by inflammatory mediators plus corticosteroids, although in a transcript-specific manner [57]. Within the brain and liver of naïve mice, Ido1-FL and Ido1-v2 expression was extremely low, similar to our previous report [57]. Ido1-FL and Ido1-v2 expression were not increased by restraint-stress in the current study; however, a previous study observed a 14-fold increase in whole-brain Ido1-FL following acute combined acoustic and restraint-stress of female BALB/c mice [39]. Whether the difference between that of Kiank and our study is due to a stronger stressor (acoustic+restraint vs. restraint), different mouse strain (BALB/c vs. C57BL/6J) or sex (female vs. male) is open to debate. Basal Ido1 expression by astrocytes from BALB/c mice may be greater than that of C57BL mice, permitting detection of Ido1 induction in whole brain by stress. Independent of this, Ido1-FL and Ido1-v2 are inducible *in vivo* by inflammatory signals such as LPS [33], [126] and Ido1-FL induction in the mouse brain by

mycobacterium infection is IFNγ-dependent [142]. Since IFNγ was not increased in our acute-stress model, Ido1-FL was not induced. Thus, our data clearly suggest that neither Ido1-FL nor Ido1-v2 mediate the stress-induced Kyn levels in the brain of C57BL/6J mice.

Ido1-v1 is the major Ido1 transcript within the naïve mouse brain, and its expression is increased by LPS *in vivo* [126] and IFNγ *ex vivo* [57]. IFNγ also induces Ido1-Tot expression in astrocytes [102] and microglia [143]. However, the current data suggest IFNγ-independent stimuli must be responsible for Ido1-v1 induction in astrocytes by stress. Although Ido-v1 was not increased by stress in whole-brain, its expression was increased by stress in the frontal cortex and hypothalamus (Figure 6), likely within astrocytes. The significance of the cortex and hypothalamus sensitivity to restraint-induced Ido1-v1 induction relative to behavior remains to be determined.

Astrocytes are a heterogeneous population of related cells [144] varying in functionality across brain-regions [145]. Previous reports assessing Ido1-Tot indicated primary cultures of murine astrocytes do not express Ido1 [102]. By contrast, we detected Ido1-v1 expression in freshly isolated astrocytes, albeit at lower levels compared to the brains from which they were isolated. Thus, the distribution of Ido1 transcripts is cell-type specific. Astrocytes are not the major source of central Ido1-v1, but a 4.8-fold induction by stress could be critical to changes in astrocyte Kyn and KynA production.

We detected minimal expression of Ido1 transcripts in liver which were unaffected by restraint-stress; however, Ohta found increased hepatic Ido1-Tot following 6 h of water-immersion restraint-stress. This was associated with increased hepatic and serum IFNγ [38].

Thus, it is possible that our acute stress was insufficient for inducing IFNγ necessary for hepatic Ido1 induction.

Acute stress increases Ido2-v3 expression in astrocytes

Ido2 expression in the brain and liver is consistent with our previous report [57]. For example, Ido2-FL is enriched in the striatum, yet its expression is highest in liver (Figure 7A). Sets of Ido2 transcripts share expression patterns: Ido2-FL and Ido2-v4 are enriched in striatum, while Ido2-v1, Ido2-v2, Ido2-v3, Ido2-v5 and Ido2-v6 are enriched in hypothalamus, but Ido2-v8 was not detected in the brain (Figure 7 and Figure 8). The cell-types responsible for these differences are unknown. Cells within distinct brain-regions must either differently splice Ido2 pre-RNA to generate different transcripts or utilize different promoter regions to initiate transcription thereby generating different transcripts. The same can be said for Ido1 and Tdo2 transcript profiles, albeit Ido2 is the most complex.

Mazarei found enriched expression of both Ido1-Tot and Ido2-Tot in striatum and that $Ido1^{KO}$ mice were deficient in Ido2-Tot within the striatum [85], reflecting our expression pattern for Ido1-v1 or Ido2-FL, Ido2-v4 and Ido2-v6, respectively (Figure 6, Figure 7 and Figure 8). Moreover, unlike wild-type mice, Ido2-v3 expression was not increased by stress in $Ido1^{KO}$ mice (Figure 7B), further highlighting the dysregulation of Ido2 within the brain in the absence of Ido1. These findings raise the issue as to whether behavioral changes seen with $Ido1^{KO}$ mice may also be dependent on reduced basal Ido2 expression and the non-inducible nature of Ido2 in $Ido1^{KO}$ mice. This is important, as Ido1 is considered anti-inflammatory, whereas Ido2 acts as a pro-inflammatory facilitator most likely via an enzymatic-independent mechanism [146].

Astrocytes and microglia also differentially express Ido2 transcripts. Ido2-v3 (Figure 7B) was the only Ido2 transcript upregulated by stress and this only occurred within astrocytes. When cloned and overexpressed, the enzymatic activity of the protein encoded by Ido2-v3 was less than that from Ido2-FL [100]. Thus, the enzymatic or non-enzymatic role that stress-responsive Ido2-v3 plays in animal behavior, brain function and Kyn production remains to be determined.

In the current study, both astrocytes and microglia were found to express all three DOs, albeit to varying degrees. However, the DOs may not be universally co-expressed. As an example, within the liver Ido2 and Tdo2 are constitutively expressed by mature hepatocytes [128], [147], whereas low but interferon-γ-inducible Ido1 expression is found within hepatic stellate cells [96]. This pattern may explain the abundant relative expression of Ido2 and Tdo2 compared to Ido1 found by liver in the current study. One of the critical issues under investigation by several laboratories is the need for this segregation. The three DOs have redundant enzymatic activity (i.e. Trp→Kyn metabolism) but they also have non-redundant functions such as Ido1-non-enzymatic mediated self-tolerance [90] and Ido2-pro-inflammatory mediation of autoimmunity [146]. Clearly in the current work, restraint-stress induces specific Ido1, Ido2 and Tdo2 transcripts within astrocytes while decreasing Ido2 and increasing Tdo2 in liver. Whether these regulatory profiles result in distinct cellular functions is unknown and warrants consideration. Compared to brain, liver greatly over-expresses Ido2-FL, Ido2-v3, Ido2-v4 and Ido2-v8, consistent with previous reports of abundant hepatic Ido2 protein [128] and mRNA [57]. In contrast, Ido2-v1, Ido2-v2, Ido2-v5 and Ido2-v6 expression levels are higher in all brain-regions compared to liver (Figure 7 and Figure 8). These data clearly illustrate the utilization of alternative-gene processing to produce distinct Ido2 transcript profiles. The ability

to fine-tune the Ido2 transcriptome endows the brain and liver with precise regulatory control for responding differentially to stressors.

Hepatic Ido2 expression did not appear to differ significantly between wild-type and Ido1^{KO} mice, although Kolodziej [84] found decreased Ido2 expression in inguinal lymph nodes of Ido1^{KO} mice. Nevertheless, stress resulted in decreased hepatic Ido2-FL, Ido2-v3 and Ido2-v4 and striatal Ido2-v1. Again, the functional consequences of these tissue-specific changes on behavior remain undetermined.

Determining the mechanism behind perturbed (diminished) expression of specific Ido2 transcripts in Ido1^{KO} mice is outside the scope of the current study. Ball *et al.* hypothesized that, since Ido1 and Ido2 are adjacent on mouse (and human) chromosome 8 (Tdo2 is on murine chromosome 3), the excision of the 3 exons and intervening introns of the Ido1 gene (Figure 3 top) may perturb Ido2 expression by disrupting cis-regulatory elements [80]. The Ido1 gene (spanning 13 kbp) is located 5' of Ido2 (spanning 22 kbp) with less than 8 kbp's separating the two genes on both the mouse and human chromosomes. While both promoters and enhancer elements are short DNA sequences (typically 100 bp and 50-1,500 bp, respectively), promoters are generally very near the transcription start site (TSS). In contrast, enhancer elements may be located far (> 100,000 bp) upstream of the TSS [148]. Thus, enhancers controlling Ido2 expression could easily reside within the upstream Ido1 gene. Thus far, we are unaware of any reports identifying upstream regulatory elements involved in Ido2 expression. However, future studies utilizing the commercially available Ido1^{KO} mouse model should consider the involvement of both altered Ido2 expression (current work and [80], [100]) and activity[100].

Similar to murine Ido2, alternative transcripts for human IDO2 have been described with transcripts lacking various exons or initiating at exon 1 or 2 [79], [149]. Human IDO2 transcripts containing exon 10 are widely expressed across tissues including liver and brain, but those amplified with a forward primer in exon 1a plus reverse primers in either exon 8 or exon 10 are limited to placenta and brain [79]. Although not confirmed by qPCR analysis, alternate transcripts for human IDO1 and TDO2 have been described [150], [151]. Thus, DO expression and regulation in humans is most likely also transcript-, tissue- and cell-specific. Characterization of human DO transcript regulation by inflammatory or stress-related signals has not been reported.

Acute stress increases Tdo2-FL expression in brain, astrocytes and liver

Our data (Figure 9A-C) support previous reports of abundant hepatic expression of Tdo2 relative to the brain [55], [57] as well as the upregulation of Tdo2 mRNA in liver by restraint-stress [38], [56] and in brain-slice cultures by glucocorticoids [57]. However, this is the first report describing the stress-induced upregulation of hepatic Tdo2-FL and Tdo2-v2 and of all three functionally characterized Tdo2 transcripts [55] within whole-brain. However, stress did not induce Tdo2 transcripts in selected brain-regions (PFC, Stri, Hippo and Hypo). Similarly, Gibney reported no acute stress-induced change in Tdo2-Tot mRNA within the PFC [56], further implicating other brain-regions such as the cerebellum or brain stem [37], [55], [85] in the central Tdo2 transcriptional response to acute restraint-stress.

Shimazu first reported increased hepatic Tdo2 activity (*in vitro* conversion of Trp to Kyn by liver homogenate) following peripheral administration of corticosterone, or by stimulation of the hypothalamic sympathetic nucleus, both effects seemingly independent of adrenal secretions. However, hepatic Tdo2 enzymatic activity was greater when the hypothalamus of

animals with intact adrenals were stimulated [58]. Indeed, the addition of the synthetic glucocorticoid dexamethasone to primary hepatocytes increases Tdo2 activity [59], [60] and mRNA levels [61], [62], [152]. Acute stress also increases hepatic Tdo2 activity [38], [56], [117], [135], an effect either reportedly requiring [135] or only partly moderated by adrenal secretions [38], [117]. Collectively, these data suggest hepatic Tdo2 activity is increased in a glucocorticoid-dependent (stress, corticosterone-induced) and glucocorticoid-independent (direct hypothalamic stimulation) manner.

Whether glucocorticoids directly mediate the upregulation of all three Tdo2 transcripts in the brain is unlikely since dexamethasone only upregulated Tdo2-FL in brain-slice cultures [57]. Thus, Tdo2-FL induction likely represents the adrenal-dependent transcript in the aforementioned studies, whereas Tdo2-v1 and Tdo2-v2 represent the adrenal/glucocorticoid-independent transcripts. Again, it appears that tissues utilize different DO transcripts to fine-tune responses to specific physiologic inputs. Defining the Tdo2 transcriptome within specific hepatic cell-types is needed to completely understand Tdo2 (and Ido) regulation in response to stress.

Following acute stress, only the glucocorticoid-responsive Tdo2-FL transcript was induced within astrocytes. Curiously, the stress-induced increase in Tdo2-FL was preserved in astrocytes of Ido1^{KO} mice but not within whole-brain homogenate of Ido1^{KO} mice (Figure 9A). Thus, although genetic deletion of Ido1 blocks stress-induced increases of astrocyte Ido2-v3 (Figure 7B), astrocyte Tdo2-FL expression is still increased by stress in Ido1^{KO} mice (Figure 9A). Hence, in astrocytes, the induction of Ido2 is Ido1-dependent, but the induction of Tdo2 is Ido1-independent. Tdo2 (unknown transcript) is present primarily in neurons and astrocytes [32]. The stress-induced increase in Tdo2-v1 and Tdo2-v2 in whole brain is not seen in astrocytes (or

microglia). Presumably, this induction is occurring within neurons and surprising requires Ido1 (Figure 9). Elevated Tdo2 in neurons and astrocytes, along with elevated KynA, is a hallmark of schizophrenia [153], [154]. Stress-induced Tdo2 may provide the comorbidity link between stress and schizophrenic development and psychosis [114], [155].

Downstream Kynurenine Pathway genes and their regulation by stress

The enzymatic machinery downstream of the DOs define the cellular-specificity of Kyn metabolism into neuroactive kynurenines [29], [32]. Astrocytes expressed significantly more Kat2 and less Kynu, Kmo and Haao than microglia (Figure 10A-D). Thus, downstream of the DOs, the relative expression levels of *Kynurenine Pathway* enzymes within astrocytes and microglia support current dogma which holds that astrocytes predominately produce KynA [32], [47], [137], [138], [156] and microglia produce 3-HK and QuinA [32], [42], [51]. Although there are 3 well-characterized Kat enzymes, Kat2 is believed to account for the majority of KynA production in the mammalian brain [157]. Whether Kat1, Kat2 or Kat3 or their mRNA isoforms are differentially regulated in the brain or liver in response to stress is not known and outside the scope of the current project.

Downstream *Kyn Pathway* enzymes are spatially and temporally regulated in the brain by inflammation [158] and acute stress [119]. Following acute stress, we found minor changes in the expression of these enzymes, such as the increase in astrocyte and microglia KMO. Nonetheless, the DOs are considered rate-limiting and DO expression was increased by acute stress in astrocytes (Figures 6-8). Both acute and chronic stress increase brain KynA [37], [125] despite unchanging Kat2 expression (Figure 10). These findings suggest that the increase in KynA is controlled by elevated astrocyte DO expression without a necessary increase in Kat2. In contrast to the mild inductions by stress, acute LPS-induced inflammatory responses cause 2 to

5 fold increases in KMO expression (dependent on brain region) without induction of Haao or Kynu in the mouse brain and small changes in Kat2 expression [158]. These inflammatory responses suggest more robust changes in microglial downstream enzymes during neuroinflammation compared to stress.

The relevance of these findings to human well-being remains conjectural. The *Kynurenine Pathway* is implicated in mediating symptoms of depression induced by chronic stress and inflammation [109]. Increased plasma Kyn and cerebral spinal fluid levels of Kyn and QuinA are associated with depression symptomology in patients treated with IFNα [159]. In adolescents, the peripheral Kyn:Trp ratio correlates with anhedonia scores [160] and increased suicidality [161]. These findings have been largely attributed to altered IDO1 activity [32] with little known regarding the role of IDO2. In contrast, evidence is building for TDO2 involvement in human mental health.

White-matter astrocyte TDO2 immune staining was increased in *post mortem* frontal cortex and anterior cingulate samples of Schizophrenic patients compared to controls [153], [162]. Schizophrenic patients have elevated salivary [48], cerebrospinal fluid [163] and frontal cortex levels of KynA [163]. Tdo2 mRNA expression in frontal cortex [162] and anterior cingulate [153] are greater *post mortem* in Schizophrenic patients than controls. One hallmark of Schizophrenia is the reduced ability of a 'prepulse' (or weak cue) to inhibit the natural startle response to a subsequent stronger stimulus [164]. Prepulse inhibition is also disrupted by increased brain KynA following peripheral administration of Kyn to rodents [165]. Within the brain, KynA reduces neurotransmitter activity by antagonizing α_7nACh [46] and NMDA receptors [42]. Acute stress increases urinary [166] and salivary [48] KynA levels in healthy volunteers. Acute stress also increases salivary KynA in Schizophrenic patients and greater KynA

levels correlated with greater disease severity [48]. However, we are unaware of studies quantifying stress effects on central levels of KynA in humans. Nonetheless, mitigating stress [48], [155] and targeting astrocyte DOs and/or KATs are proposed as a therapeutic approaches for treating Schizophrenia [109], [112], [154]. While astrocyte Tdo2 is stress-sensitive in rodents and implicated in mediating increased central KynA in patients and rodent models of Schizophrenia, the expression of alternative DO transcript in humans following stress and in the pathogenesis of Schizophrenia remain totally un-defined.

Conclusions

Although several studies have implicated the *Kynurenine Pathway* in stress-induced depression-like behavior, the cellular-origin and transcript-specificity of the DOs remained undefined. Herein, we report that all three DOs were upregulated by stress in a cell- and transcript-specific manner. Specifically, Ido1-v1, Ido2-v3 and Tdo2-FL were all increased in astrocytes. In contrast, brain Tdo2-v1 and Tdo2-v2 were upregulated by stress, a response that is independent of astrocyte or microglial expression. When only investigating whole-brain or brain-regions these subtle but critical changes are easily overlooked. Remarkably, stress did not increase several DO transcripts in brain of $Ido1^{KO}$ mice, suggesting an Ido1 requirement for their induction. Thus, our data highlight a significant perturbation of Ido2 and Tdo2 regulation within the brain of $Ido1^{KO}$ mice. The specific role for astrocytes in acute stress is supported by reports of stress-induced increases in central kynurenic acid (KynA) [37], [39], [125], the major downstream *Kynurenine Pathway* product produced by astrocytes [44], [137], [138]. Future studies examining the effect of stress on the *Kynurenine Pathway* should consider the role of all three DOs and examine cell-specific changes. Undoubtedly, a single qPCR assay will frequently miss cell-specific changes in DO expression.

CHAPTER 3: Glia- and tissue-specific changes in the *Kynurenine Pathway* after treatment of mice with lipopolysaccharide and dexamethasone [2]

3.1 Abstract

Behavioral symptoms associated with mood disorders have been intimately linked with immunological and psychological stress. Induction of immune and stress pathways is accompanied by increased tryptophan entry into the Kynurenine Pathway as governed by the rate-limiting enzymes indoleamine/tryptophan 2,3-dioxygenases (DOs: Ido1, Ido2, Tdo2). Indeed, DO expression is associated with inflammation- and stress-related depression symptoms. Here we examined central (whole brain, astrocyte and microglia) and peripheral (lung, liver and spleen) DO expression in C57BL/6J mice treated intraperitoneally with lipopolysaccharide (LPS), dexamethasone (DEX) or LPS+DEX to model the response of Kynurenine Pathway enzymes to inflammation and stress. LPS-induced expression of interferon-(Ifn)γ and tumor necrosis factor (Tnf)α was attenuated by DEX, confirming inflammatory and anti-inflammatory responses to LPS and DEX, respectively. Increased kynurenine levels following LPS and DEX administration verified increased Kynurenine Pathway activity. The expression of multiple isoforms for each DO were quantified. LPS increased Ido1-FL expression in the mouse brain (~1,000-fold), a response paralleled by increased Ido1-FL expression by both astrocytes and microglia. Central Ido1-FL expression was not changed by DEX; however, LPS-induced Ido1-FL was decreased by DEX in peripheral tissues. In contrast, DEX increased Ido1-v1 expression by astrocytes and microglia, but not by peripheral tissues. In

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[2] Edited from original submitted manuscript with headings, figures, and tables renumbered:
Dostal CR, Gamsby NS, Lawson MA, M^{C}Cusker RH. Glia- and tissue-specific changes in the *Kynurenine Pathway* after treatment of mice with lipopolysaccharide and dexamethasone. *in Review*

comparison to Ido1, brain Ido2 was minimally induced by LPS or DEX. Uniquely, Ido2-v6 was LPS- and DEX-inducible only in astrocytes suggesting a unique role for astrocytes in the DO response to inflammation and stress. Three Tdo2 isoforms were quantified. DEX increased Tdo2-FL expression in brain and astrocytes (not microglia) while LPS failed to induce central Tdo2 expression. However, peripheral Tdo2 isoforms were upregulated by LPS and DEX with isoform- and tissue-specificity. In summary, expression of specific DO isoforms is increased by both LPS and DEX, but LPS-dependent Ido1 and Ido2 induction are attenuated by DEX only in the periphery. These findings demonstrate a plausible interaction between immune activation and glucocorticoids associated with depression.

3.2 Introduction

The brain and immune system have a sophisticated bidirectional relationship that not only provides protection for the brain, but also affords a mechanism whereby pro-inflammatory cytokines influence mood and behavior. Under most conditions, this action allows appropriate immune responses to physiological stressors. However, when inappropriate responses occur, major psychiatric sequelae may emerge [29]. Similar to immune-related psychiatric changes, the hypothalamic pituitary adrenal (HPA) axis response occurring during immune activation and following psychological stress is intimately related to mental wellbeing [29], [167]. Depression is a prime example. Major depression, the most common psychiatric disorder in the United States, presents more often as a co-morbidity to other illnesses rather than alone [107], [108]. In fact, major depression is frequently associated with elevated corticosteroid levels resulting from dysfunction of the HPA axis [168]–[170] and elevated levels of pro-inflammatory cytokines [171]–[173]. Further defining the relationship between the brain and the immune system

should aid in the development of new treatments for depression and other psychiatric disorders [174].

Physiological challenges such as infection, cancer, heart disease or perceived psychological stress activate the immune system and increase HPA axis activity [175]. In turn, resultant pro-inflammatory cytokine and adrenocortical secretions induce the *Kynurenine Pathway* [54]. Evidence continues to accumulate implicating increased levels of tryptophan metabolites (i.e. kynurenines) in precipitating or mediating depression symptomology [32], [109], [111], [161], [176]–[178]. Tryptophan (Trp) entry into the *Kynurenine (Kyn) Pathway* is governed by the indoleamine and tryptophan 2,3-dioxygenases (DOs: Ido1, Ido2, Tdo2). It is currently accepted that Ido1 and Ido2 are upregulated by pro-inflammatory cytokines, whereas Tdo2 is controlled by corticosteroids [54]. Although inflammatory cytokines and stress-induced corticosteroids may individually contribute to disease morbidity and mortality via DO upregulation [109], [179], [180], the interacting effects of inflammatory mediators and glucocorticoids on DO expression remain poorly defined.

Increased DO expression results in increased Trp → Kyn metabolism and the subsequent production of downstream kynurenines (Kyn's). An underappreciated aspect of this field is the cell-specific generation of unique Kyn's with opposing effects [32]. In the brain for example, quinolinic acid (QuinA) generated predominantly by microglia is a glutamate receptor (N-methyl-D-aspartate, NMDA-R) agonist while kynurenine acid (KynA) generated by neurons and astrocytes is a NMDA-R antagonist [54]. In addition to their neuromodulatory functions, Kyn's are also immunomodulatory largely by the ability of Kyn and KynA to bind and activate the aryl hydrocarbon receptor and suppress the immune response [31]. Thus, an imbalance in Kyn's is implicated in various illnesses including neurodegenerative disorders [181], [182], autoimmune

diseases [101], [183]–[185], cancer [95], [132], [179] and psychiatric conditions such as schizophrenia [48], [112], [186] and depression [111], [159], [176], [178], [187].

Data generated with animal models demonstrate that inflammation- or stress-induced depression-like behaviors and cognitive deficits are DO-dependent [33], [35], [56], [67], [68], [125]. Both inflammation and stress increase DO activity in the central nervous system and peripheral tissues. Thus, we probed the interacting effects of LPS (an immune system activator used to model an acute inflammatory response) and DEX (a glucocorticoid receptor agonist used to model an acute stress response) on DO mRNA expression in both central (brain, astrocytes and microglia) and peripheral (liver, lung and spleen) samples.

3.3 Methods

Animals

Male C57BL/6J mice (Bar Harbor, ME, USA) kept on a reversed 12 h light-dark cycle with *ad libitum* access to food and water were individually housed 3 weeks prior to experiments. Mice were handled for 5 consecutive days prior to treatment and were 15 weeks of age at the time of treatment. All animal procedures were approved by the Institutional Animal Care and Use Committee and performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council).

Intraperitoneal (i.p.) treatments

Mice were injected just prior to onset of the dark cycle with either saline (Control) or saline containing lipopolysaccharide (LPS; 0.83 mg/kg body weight final dosage, serotype 0127:B8, Sigma), dexamethasone (DEX; 10 mg/kg body weight final dosage, 11695-4013-1, Henry Shein) or a single injection to deliver LPS+DEX. Mice were euthanized by CO_2

asphyxiation 5 h after treatment. To confirm treatment effectiveness associated with this short treatment interval, changes in body weight and food intake were quantified. Statistical analysis indicated that both LPS ($p<0.05$) and DEX ($p<0.05$) reduced body weight (Δ body weight: Control 0.2±0.1 g, LPS -1.3±0.1 g, DEX -0.2±0.1 g, LPS+DEX -2.2±0.2 g), but only LPS ($p<0.05$) reduced 5 h food intake (Control 1.3±0.1 g, LPS 0.2±0.2 g, DEX 1.6±0.2 g, LPS+DEX 0.1±0.0 g).

Glia enrichment (cohort 1)

After a 5 h treatment and following intracardial perfusion with 30 ml of ice-cold PBS containing 2 mM EDTA, brains were removed and gently homogenized. Whole brain homogenates were sampled prior to the enrichment of astrocytes and microglia by magnet-assisted cell separation (Miltenyi Biotec Inc.) as recently described [86]. Brain homogenate, microglia and astrocyte samples were suspended in TRIzol and stored at -80 °C until processed for assessment of gene expression.

Tissue collection (cohort 2)

After a 5 h after treatment, blood was collected from the inferior vena cava immediately prior to intracardial perfusion with ice-cold PBS. After perfusion, brain and peripheral tissues (liver, lung, spleen) were harvested, immediately frozen in liquid N_2 and then stored at -80 °C. Brain and plasma were processed as previously reported for Kyn content [86]. Peripheral tissues from this cohort of mice were processed for gene expression.

Gene expression by qPCR

Methods for RNA extraction, reverse-transcription and analysis of quantitative polymerase chain reaction (qPCR) data were recently described in detail [86]. Within each tissue or cell-type, the expression of each test gene was normalized to Gapdh using the $2^{-\Delta\Delta Ct}$

method, C_t = cycle threshold [131]. Gene expression in the Control group was set to 1.0 and other treatment groups are expressed as fold expression relative to Control. DO gene structure and known mRNA transcripts were previously described [86]. PCR probe-based assays were purchased from IDT (Coralville, Iowa) and custom assays were designed using the IDT PrimerQuest® Design Tool. Because of low expression, some transcripts are 'not detectable' in all samples (i.e. C_t values 'undetermined') thus preventing calculation of relative gene expression; for analysis, a C_t value of 40.0 is assigned when this occurs.

HPLC

To measure Kyn, brain and plasma samples were analyzed by high pressure liquid chromatography (HPLC) with electrochemical detection using methods recently described in detail [86].

Statistics

Data are presented as mean ± SEM representing 4-6 mice per treatment group. Two-way ANOVA was performed for all data. Significance was set at $p \leq 0.05$. In the absence of a statistical interaction between treatments, significant main effects of LPS or DEX are annotated with * or ϕ, respectively. In the presence of a significant statistical interaction between LPS and DEX, data are annotated with δ to represent significant effects by *post hoc* analysis using the Holm-Šídák method. Graphic presentation and statistical analysis were completed using SigmaPlot 13.0 (Systat Software, Inc., San Jose, CA).

3.4 Results

DEX is anti-inflammatory in peripheral tissues when co-administered with LPS

FK506 binding protein 5 (Fkbp5) expression was assessed to confirm glucocorticoid-receptor mediated responses [133], [134]. Fkbp5 is well expressed by all 4 tissues (Figure 11, C_t of Controls (Ctrl/-) presented above each graph, for each tissue). In brain, Fkbp5 expression was increased 3.4-fold by LPS and 5.9-fold by DEX relative to Controls. The effect of LPS indicates inflammation-dependent induction of the HPA axis [188]. DEX also increased Fkbp5 expression in liver, lung and spleen relative to Control. LPS caused a smaller increase in Fkbp5 expression in liver and lung when compared to DEX, but LPS did not increase Fkbp5 in spleen. Co-treatment of LPS+DEX did not result in greater Fkbp5 within these peripheral tissues compared to either treatment alone, suggesting saturation of the peripheral glucocorticoid response by DEX alone.

Ifnγ and Tnfα expression were quantified to determine relative ability of LPS and DEX to modulate an inflammatory response. Ifnγ expression was not detected in brains (Figure 11B, see Control C_t value) with the highest expression measured in spleen. LPS did not induce Ifnγ expression in the brain, but increased Ifnγ expression in liver, lung and spleen (Figure 11B). The increase in Ifnγ expression caused by LPS was abrogated by co-administration of DEX in all three peripheral tissues. Tnfα expression in brain, liver, lung and spleen was elevated by LPS (Figure 11C). LPS-induced Tnfα was significantly attenuated by DEX co-treatment for all three peripheral tissues, but not in the brain.

Together these data indicate HPA axis activation by LPS with a smaller glucocorticoid-like effect than DEX administration. In contrast, LPS induces an inflammatory response that is

antagonized by DEX, but only in the periphery. These data provide strong evidence of treatment efficacy.

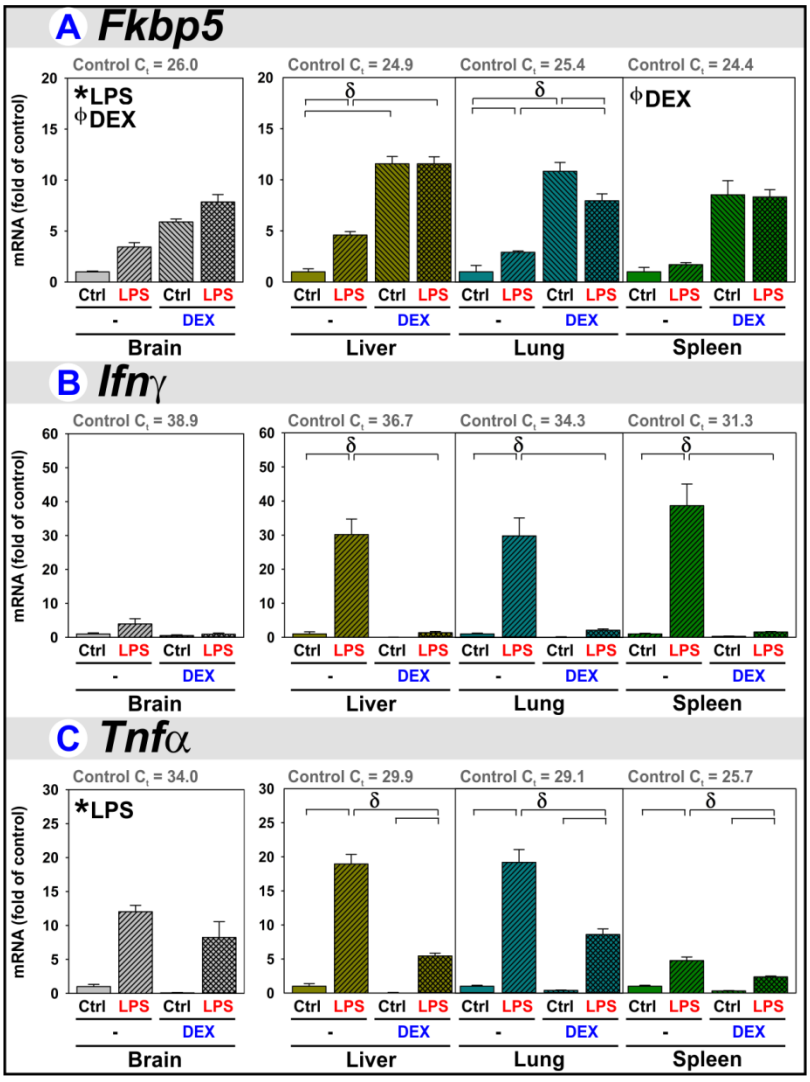

Figure 11. DEX is anti-inflammatory in peripheral tissues when co-administered with LPS. Perfused brain, liver, lung and spleen tissues were obtained from mice 5 h after i.p. treatment with saline (Control), LPS, DEX, or LPS+DEX to assess HPA activity by quantifying **(A)** Fkbp51 expression and to assess an inflammatory response by quantifying **(B)** Ifnγ and **(C)** Tnfα expression. * $p < 0.05$ main effect of LPS. $^{\phi}$ $p < 0.05$ main effect of DEX. $^{\delta}$ $p < 0.05$ *post hoc* mean separation (brackets) in the presence of a significant LPS x DEX interaction.

Kyn levels increase in brain following LPS and in plasma following DEX

Kyn was quantified to confirm *Kynurenine Pathway* activation. Plasma Kyn (Figure 12A) was elevated ~50% following DEX administration. LPS did not increase plasma Kyn, but prevented the increase triggered by DEX. In contrast, brain Kyn (Figure 12B) was doubled by

LPS. DEX did not change brain Kyn but prevented the increase produced by LPS. These data strongly illustrate independent increases in *Kynurenine Pathway* activity in the periphery and brain following DEX and LPS treatments, respectively.

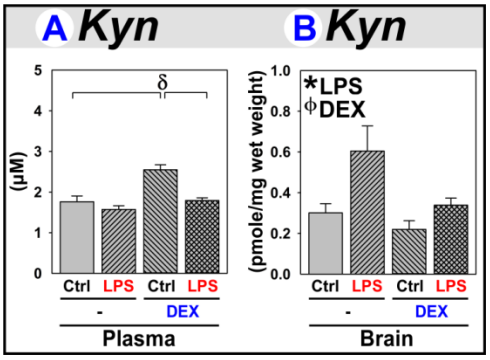

Figure 12. Kyn levels increase in brain following LPS and in plasma following DEX. (A) Plasma and **(B)** brains were obtained from mice 5 h after i.p. treatment with saline (Control), LPS, DEX, or LPS+DEX to assess Kyn levels by high pressure liquid chromatography. * $p < 0.05$ main effect of LPS. $^{\phi}$ $p < 0.05$ main effect of DEX. $^{\delta}$ $p < 0.05$ *post hoc* mean separation in the presence of a significant LPS x DEX interaction.

LPS and DEX differentially regulate Ido1 expression in brain, glia and peripheral tissues

Expression of the reference full-length Ido1 transcript (Ido1-FL: NM_008324.2), two other protein coding mRNA isoforms (Ido1-v1: NM_001293690.1 and Ido1-v2: XM_006509018.1) and their combined expression (Ido1-Tot) were quantified.

Ido1-FL is poorly expressed in naïve brain, astrocytes and microglia (Figure 13A, see Control C_t values). Even so, Ido1-FL is strongly induced 5 h after LPS treatment. DEX did not alter Ido1-FL expression in these samples, either alone or in combination with LPS. These data strongly suggest that a large portion of the brain's response to LPS reflects Ido1-FL induction within both astrocytes and microglia. Although several publications describe Ido1 induction by microglia [189]–[191], our data clearly show that astrocytes respond robustly to LPS administration (numerically a greater fold-increase than microglia). In the periphery, Ido1-FL is expressed best by lungs, followed by spleens and livers from naïve mice (see Control C_t values).

LPS increased Ido1-FL expression in liver, lung and spleen, but DEX alone was ineffective. LPS-induced Ido1-FL expression was significantly reduced by DEX co-administration in all three peripheral tissues. Thus, Ido1-FL is responsive to inflammatory stimuli across many tissues. Interestingly, LPS-induced Ido1-FL is abrogated by DEX only in peripheral tissues.

Ido1-v1 is the only Ido1 isoform appreciably expressed in naïve mouse brains (Figure 13B, Control C_t = 33.1). Ido1-v1 expression in whole brain was unaltered by LPS or DEX. However, LPS increased Ido1-v1 expression in astrocytes, but not microglia. Ido1-v1 expression by astrocytes, and less so by microglia, was DEX-inducible compared to Controls (Figure 13B). Elevated Ido1-v1 expression by glia without noteworthy changes in brain tissue might seem inconsistent at first glance. However, Ido1-v1 expression in Control astrocytes and microglia is lower than brain (a brain C_t of 33.1 represents ~13-fold greater expression compared to the astrocyte C_t of 36.8 and ~42-fold greater expression compared to the microglia C_t of 38.5). These data indicate that most Ido1-v1 in brain is not derived from these glia, but from a LPS and DEX insensitive cell-type(s). Thus, the increase in astrocyte and microglial Ido1-v1 expression was insufficient to impact brain expression because of their lower expression levels. In peripheral tissues, Ido1-v1 was not elevated by DEX and LPS only increased Ido1-v1 in the lung where its induction was attenuated by DEX co-treatment. LPS also slightly decreased Ido1-v1 expression in the spleen independently of DEX. Thus, DEX increased Ido1-v1 expression within astrocytes and microglia, but not in whole brain or any peripheral tissue examined. This indicates that detecting physiologically relevant changes in Ido1-v1 may require examination of each tissues individual component cell-types.

Ido1-v2 expression (Figure 13C) was essentially absent in brain, astrocytes, microglia, liver or spleen. Only in lung was Ido1-v2 detected. Ido1-v2 was strongly induced by LPS in lung

where its expression was attenuated by DEX. Thus, among the tissues tested, the lung is unique in its ability to express Ido1-v2.

Ido1-Tot expression (Figure 13D) detects all three Ido1 isoforms, but its expression in Control brains reflects the expression of Ido1-v1 (Figure 13B & D, Control C_t's both ≈ 33). In brain, LPS increases Ido1-Tot expression ~10-fold, a marked attenuation relative to the ~1,000-fold increase in Ido1-FL. Thus, the fold-change in Ido1-FL overestimates the change in total brain Ido1 expression. Moreover, the ability of DEX to increase Ido1 expression by astrocytes and microglia is overlooked when quantifying Ido1-Tot since only Ido1-v1 is affected by DEX. In liver, Ido1-Tot expression mimics Ido1-FL as only this isoform is induced by LPS and inhibited by DEX. In contrast, within the lung there are no differentially expressed Ido1 isoforms; therefore, Ido1-Tot expression reflects changes in any particular isoform or the sum of all 3 isoforms. In the spleen, the Ido1 isoforms are differentially responsive to treatment; changes in Ido1-Tot reflect LPS-induced Ido1-FL as tempered by reduced Ido1-v1. The differential regulation of splenic Ido1-FL and Ido1-v1 likely reflect increases of Ido1-FL expression in one cell-type and decreases in Ido1-v1 expression by another, but exploring this possibility was outside the scope of the current research.

Together (summarized in Table 2), these data demonstrate transcript-, tissue- and cellular-specificity of Ido1 regulation using models of inflammation and stress. Although DEX has a net ability to temper Ido1 induction in the periphery, the overall effect within the brain is unchanged Ido1-FL and increased Ido1-v1 expression. These data also demonstrate that astrocytes and microglia are major expressers of LPS-induced Ido1-FL but not major sources of Ido1-v1 in the brain. Remarkably, Ido1 expression is induced in these glia by either LPS or DEX

administration in an isoform-specific manner. The three murine isoforms are differentially regulated *in vivo* and thus Ido1-Tot does not necessarily reflect their individual changes.

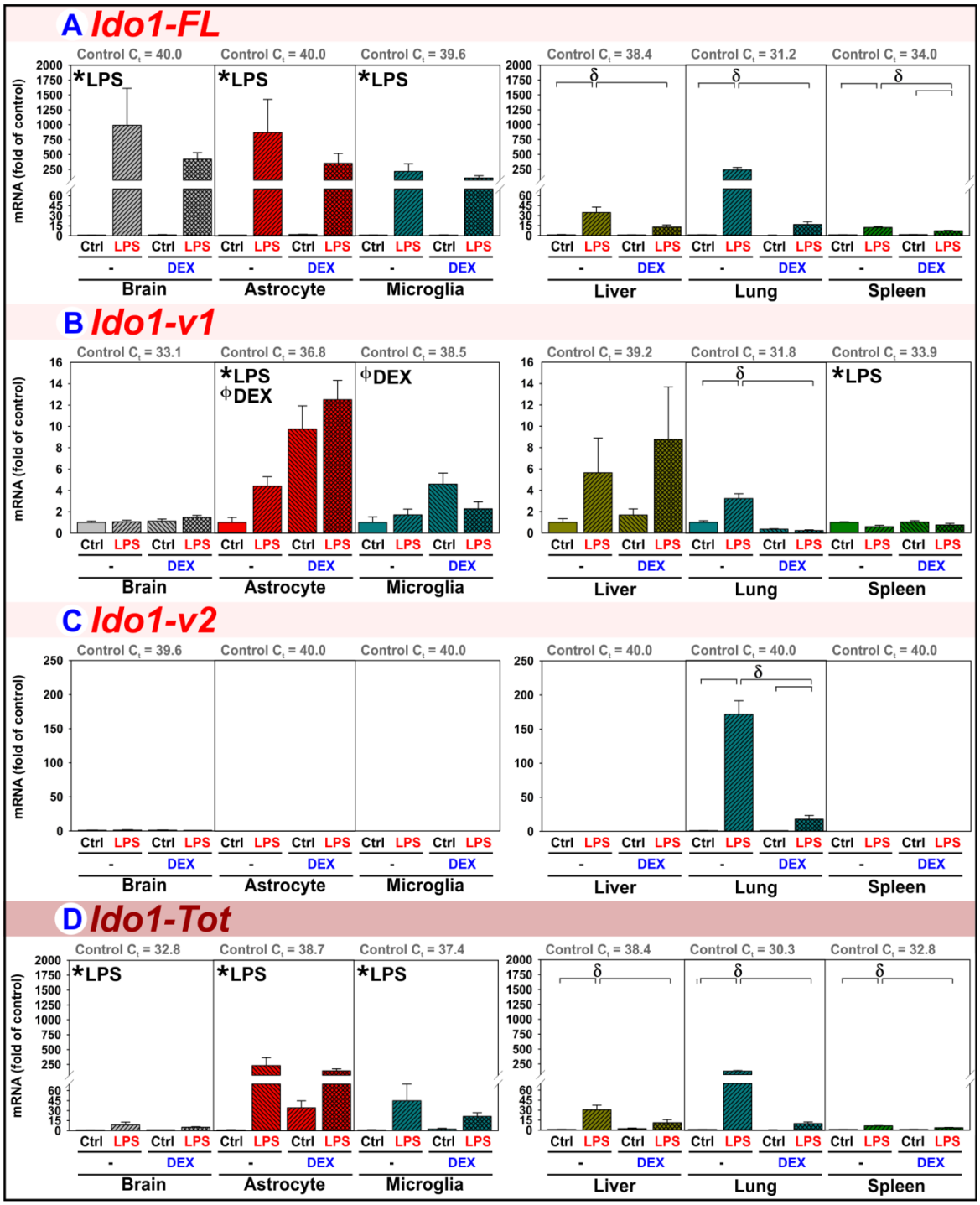

Figure 13. LPS and DEX differentially regulate Ido1 expression in brain, glia and peripheral tissues. Brain, astrocytes, microglia, liver, lung and spleen were obtained from mice 5 h after i.p. treatment with saline (Control), LPS, DEX or LPS+DEX to assess the relative changes in the mRNA expression of **(A)** Ido1-FL, **(B)** Ido1-v1, **(C)** Ido1-v2 and **(D)** Ido1-Tot. * $p < 0.05$ main effect of LPS. $^{\Phi}$ $p < 0.05$ main effect of DEX. $^{\delta}$ $p < 0.05$ *post hoc* mean separation in the presence of a significant LPS x DEX interaction.

LPS and DEX regulate Ido2 transcripts in a manner distinct from Ido1

Since murine Ido2 demonstrates transcript-specific regulation [86], [126], [190], eight variant (v) isoforms (Ido2-v1 (XM_006509048), Ido2-v2 (XM_0011242130), Ido2-v3 (Δ4, [100]), Ido2-v4 (XM_006509046), Ido2-v5 (XM_006509047), Ido2-v6 (EU440733.1), Ido2-v7 (AK082385.1), Ido2-v8 (XM_01124131)), the reference transcript Ido2-FL (NM_145949.2) and their combined expression (Ido2-Tot) were quantified to define cell- and tissue-specific expression profiles.

Ido2-v1 expression is greater in brain compared to astrocytes, microglia and peripheral tissues (Figure 14A, see Control C_t values). Ido2-v1 expression by brain is unchanged following LPS or DEX treatments. However, LPS increases Ido2-v1 expression 17-fold in astrocytes and 5-fold in microglia. The LPS induction of glial Ido2-v1 is not adequate to change whole brain expression because of low basal glial expression levels relative to brain. These data indicate that astrocytes and microglia are not major sources of brain Ido2-v1. An LPS/DEX-insensitive cell-type(s) must be the primary source of brain Ido2-v1 (mimicking Ido1-v1). In the periphery, LPS increases expression of Ido2-v1 in liver, lung and spleen. DEX alone does not change hepatic Ido2-v1 expression, abrogated the LPS effect in lung, but synergized with LPS to further elevate Ido2-v1 in spleen. Thus, Ido2-v1 expression is LPS and DEX responsive but in a cell-type and tissue-specific manner.

Ido2-v2 expression is similar in brain, astrocytes, microglia and liver, all with greater expression than lung or spleen (Figure 14B, see Control C_t values). These data indicate these glia are a major source of central Ido2-v2. Ido2-v2 expression in brain was not changed by either LPS or DEX alone but slightly elevated by LPS+DEX. This induction is not evident within astrocytes or microglia. By contrast, Ido2-v2 expression is diminished in liver by LPS, unchanged

in lung, but increased in spleen by LPS and DEX. Thus, Ido2-v2 expression is relatively stable in brain and glia, but regulation in the periphery is tissue-specific.

Ido2-v3 is better expressed by brain when compared to astrocytes and microglia, but in the periphery its expression is greatest in the liver followed by spleen and lowest in lung (Figure 14C, C_t values). These data illustrate that neither astrocytes nor microglia are the major source of central Ido2-v3. Ido2-v3 expression is slightly increased by LPS in brain and by LPS+DEX in microglia, but not astrocytes. LPS has opposing effects on Ido2-v3 expression in liver and spleen, decreasing hepatic expression and increasing splenic expression (somewhat mimicking Ido2-v2). LPS induces Ido2-v3 expression in the lung, an effect completely blocked by DEX. Thus, Ido2-v3 expression is relatively stable in brain and astrocytes, albeit inducible in microglia, but LPS and DEX responsiveness in the periphery occurs again in a tissue-specific manner.

Ido2-v6 expression is greatest in the brain, but poorly expressed in astrocytes, microglia, liver, lung and spleen (Figure 14D, C_t values). These data indicate that most of the Ido2-v6 in brain is not within these glia. Ido2-v6 expression in brain and microglia is unchanged by treatments, whereas astrocyte Ido2-v6 expression is increased by LPS and DEX. LPS also increases Ido2-v6 expression in liver, lung and spleen. DEX co-treatment abrogated LPS-induced expression in liver and lung, but by itself increased Ido2-v6 expression in spleen. Thus Ido2-v6 expression is relatively stable in brain and microglia, albeit inducible in astrocytes, but LPS and DEX responsiveness in the periphery occurs in a tissue-specific manner.

Ido2-v5 expression was only detected in brain, where it was increased by LPS (Figure 14E). Ido2-FL (the major hepatic isoform), Ido2-v4, Ido2-v7 and Ido2-v8 were only detected in

liver, where their expression levels were all decreased by LPS independently of DEX (Figure 14F-I).

Ido2-Tot expression (Figure 14J) represents the integrated effects of LPS or DEX on the multiple Ido2 mRNA isoforms. Alone, however, Ido2-Tot expression does not adequately define the elaborate isoform-specific regulation of Ido2 by LPS and DEX. For example, Ido2-Tot does not reflect the ability of LPS to induce Ido2-v1 and Ido2-v6 in astrocytes or Ido2-v1 in liver. Like Ido1, Ido2 isoform expression is likely cell-type specific within each tissue and work similar to our comparison of brain vs. astrocyte vs. microglia is needed to adequately define Ido2 regulation.

Therefore (summarized in Table 2), Ido2 exhibits regulation by LPS and DEX. These data suggest that astrocytes and microglia are not the major source of Ido2 in the brain, even after LPS or DEX treatments. Collectively, these data define distinct regulatory profiles for Ido2 transcripts.

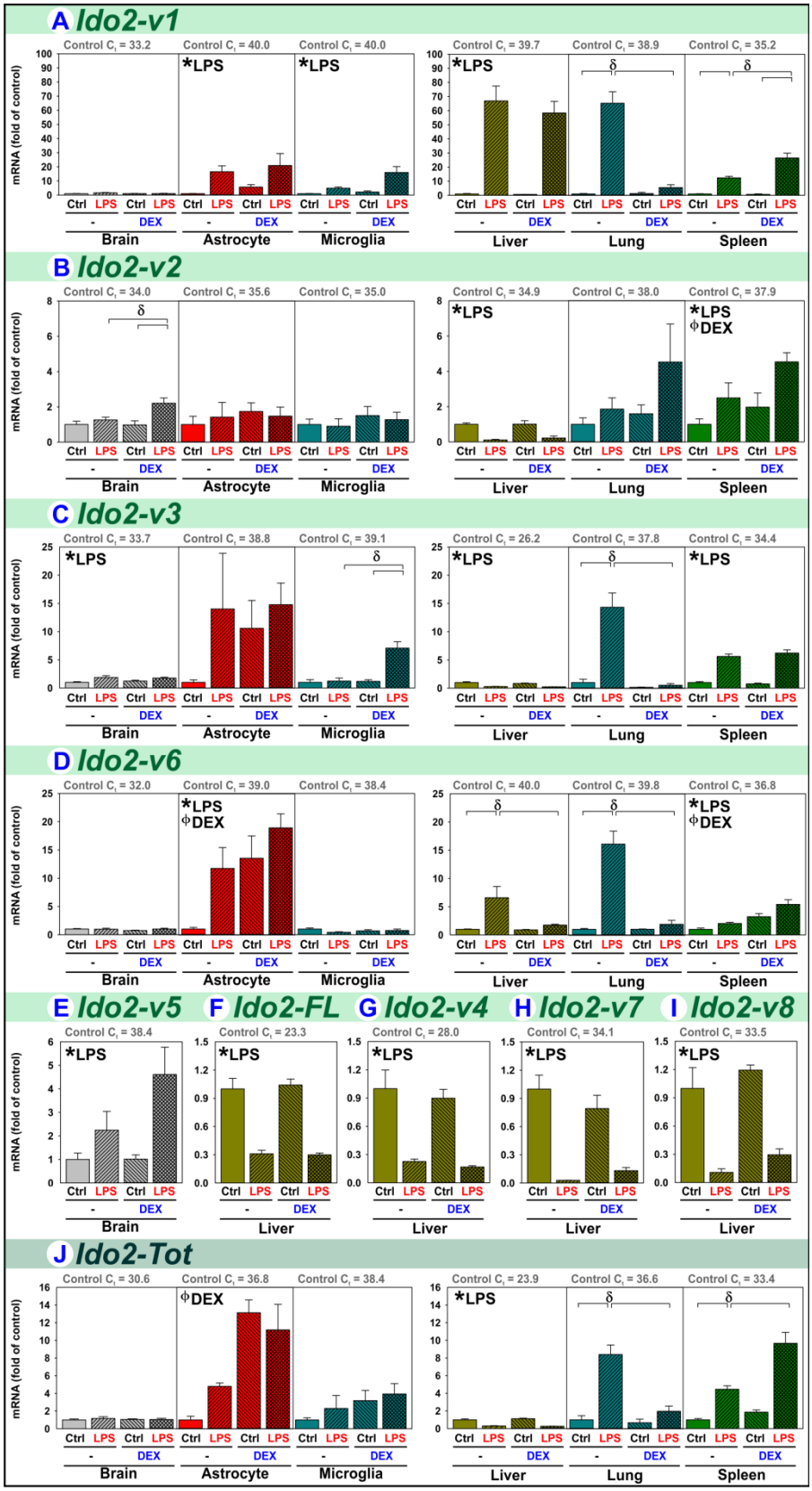

Figure 14. LPS and DEX regulate Ido2 transcripts in a manner distinct from Ido1. Brain, astrocytes, microglia, liver, lung and spleen were obtained from mice 5 h after i.p. treatment with saline (Control), LPS, DEX or LPS+DEX to assess the relative changes in the mRNA expression of **(A)** Ido2-v1, **(B)** Ido2-v2, **(C)** Ido2-v3, **(D)** Ido2-v6, **(E)** Ido2-v5, **(F)** Ido2-FL, **(G)** Ido2-v4, **(H)** Ido2-v7, **(I)** Ido2-v8 and **(J)** Ido2-Tot. * $p < 0.05$ main effect of LPS. $^{\Phi}$ $p < 0.05$ main effect of DEX. $^{\delta}$ $p < 0.05$ *post hoc* mean separation in the presence of a significant LPS x DEX interaction.

DEX upregulates Tdo2-FL while inflammation regulates Tdo2-v1 and Tdo2-v2

Tdo2 expression is widely viewed as glucocorticoid-inducible. Thus, we quantified the expression of the three known Tdo2 isoforms: the reference isoform Tdo2-FL (NM_019911.2), Tdo2-v1 (AB476989.1), Tdo2-v2 (AB476990.1) and their combined expression (Tdo2-Tot) after i.p. treatment with LPS and DEX.

Tdo2-FL is well expressed by brain and astrocytes, but less so by microglia. However, expression is highest in liver and lowest in spleen (Figure 15A, C_t values). Tdo2-FL expression increases in brain and astrocytes following DEX administration, but not in microglia. Tdo2-FL also increases after DEX administration in all three peripheral tissues. LPS only induces Tdo2-FL expression in liver. Thus, as expected from previous work, Tdo2-FL expression is glucocorticoid-dependent. The ability of LPS to increase liver Tdo2-FL may reflect HPA axis activation and subsequent glucocorticoid release and action.

Tdo2-v1 and Tdo2-v2 expression are similarly expressed and regulated. Tdo2-v1/v2 are best expressed by the liver, next highest in astrocytes, then slightly lower in microglia, lung, brain and spleen (Figure 15B-C, C_t values). Thus, astrocytes and microglia are major sources of central Tdo2-v1/v2. Their expression is unchanged by LPS and DEX in brain, astrocyte and microglia, but regulated in a tissue-specific manner within the periphery. DEX increased Tdo2-v1/v2 expression in liver and lung, but not spleen. In contrast, LPS increases Tdo2-v1/v2 expression in liver and spleen, but not lung. Interestingly, LPS-inducible splenic Tdo2-v1/v2 expression was blocked by DEX. Thus, Tdo2-v1/v2 expression by liver and lung is glucocorticoid-inducible, but their expression is LPS sensitive in liver and spleen.

Tdo2-Tot (Figure 15D) expression represents a composite of the three Tdo2 isoforms. In brain, Tdo2-Tot is not changed by LPS or DEX. When using this assay, the stimulatory effect of DEX on brain Tdo2-FL is completely missed because Tdo2-Tot also detects the two unresponsive isoforms. With astrocytes and microglia, Tdo2-Tot expression reflects Tdo2-FL induction by astrocytes and unchanged isoform expression in microglia. In liver, all Tdo2 isoforms are induced by both LPS and DEX; this is reflected by Tdo2-Tot expression. Similarly, in lung all Tdo2 isoforms are DEX inducible as is Tdo2-Tot. However, in spleen Tdo2-Tot expression is problematic. Splenic Tdo2-Tot is not significantly changed by LPS or DEX, completely overlooking the ability of DEX to induce Tdo2-FL and LPS to induce Tdo2-v1/v2. The differential expression of Tdo2 isoforms is likely a cell-type specific phenomenon and overlooked when global Tdo2 expression is quantified.

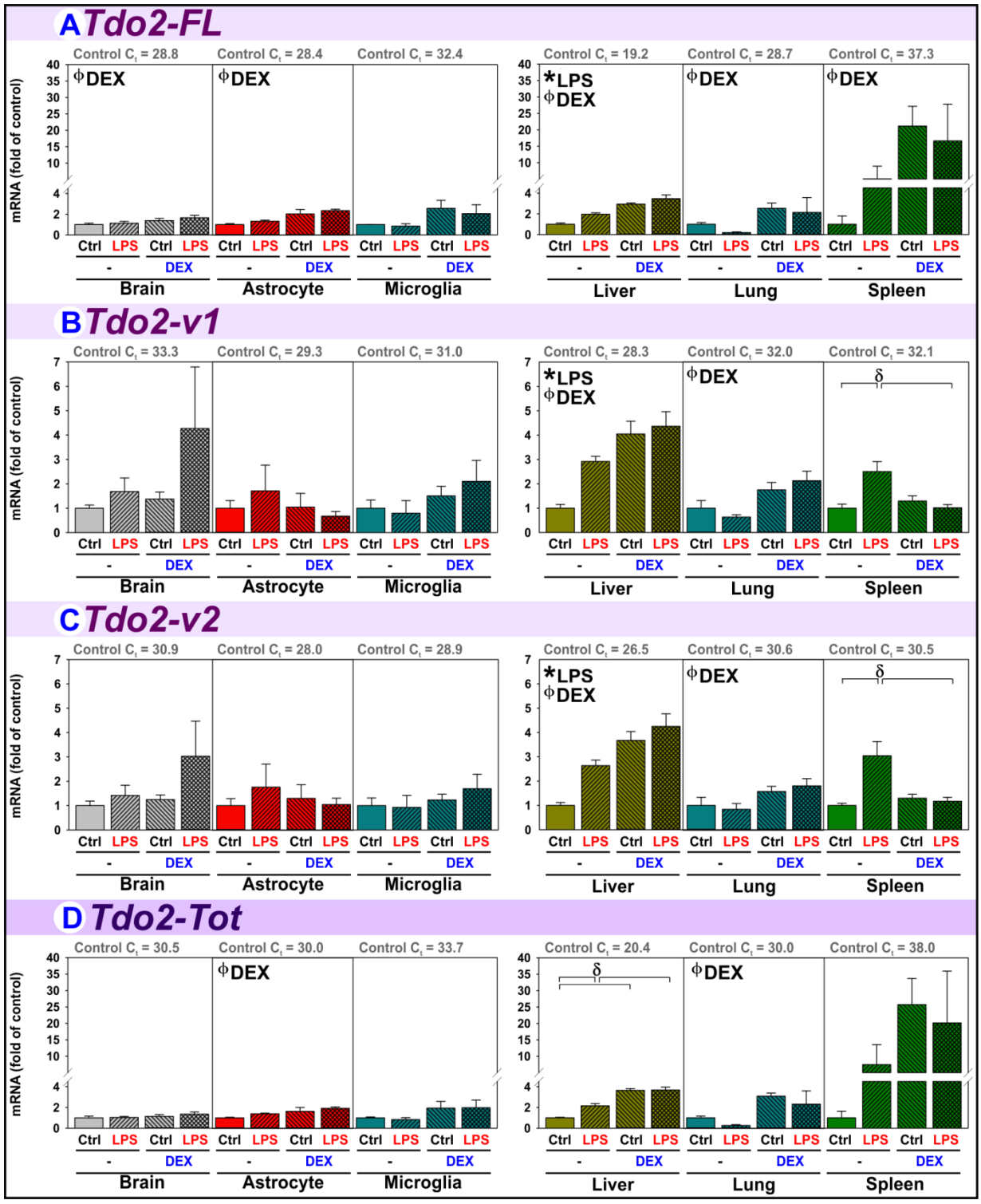

Figure 15. DEX upregulates Tdo2-FL while inflammation regulates Tdo2-v1 and Tdo2-v2. Brain, astrocytes, microglia, liver, lung and spleen were obtained from mice 5 h after i.p. treatment with saline (Control), LPS, DEX or LPS+DEX to assess the relative changes in the mRNA expression of **(A)** Tdo2-FL, **(B)** Tdo2-v1, **(C)** Tdo2-v2 and **(D)** Tdo2-Tot. * $p < 0.05$ main effect of LPS. $^{\Phi}$ $p < 0.05$ main effect of DEX. $^{\delta}$ $p < 0.05$ *post hoc* mean separation in the presence of a significant LPS x DEX interaction.

Table 2. Summary of changes in DO expression.

	brain		astrocyte		microglia		liver		lung		spleen	
	LPS	DEX	LPS	DEX	LPS	DEX	LPS	DEX	LPS	DEX	LPS	DEX
Ido1-FL	↑↑↑	-	↑↑↑	-	↑↑↑	-	↑ ·	↓	↑↑ ·	↓	↑ ·	↓
Ido1-v1	-	-	↑	↑	-	↑	↑	-	↑ ·	↓	↓	-
Ido1-v2	ND	ND	ND	ND	ND	ND	ND	ND	↑↑ ·	↓↓	ND	ND
Ido1-Tot	↑	-	↑	-	↑	-	↑ ·	↓	↑↑ ·	↓	↑ ·	↓
Ido2-FL	ND	ND	ND	ND	ND	ND	↓	-	ND	ND	ND	ND
Ido2-v1	-	-	↑	-	↑	-	↑	-	↑ ·	↓	↑ ·	↑
Ido2-v2	↑ ·	↑	-	-	-	-	↓	-	-		↑	↑
Ido2-v3, Δ4	↑	-	-	-	↑ ·	↑	↓	-	↑ ·	↓	↑	
Ido2-v4	ND	ND	ND	ND	ND	ND	↓	-	ND	ND	ND	ND
Ido2-v5	↑	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ido2-v6	-	-	↑	↑	-	-	↑ ·	↓	↑ ·	↓	↑	↑
Ido2-v7	ND	ND	ND	ND	ND	ND	↓	-	ND	ND	ND	ND
Ido2-v8	ND	ND	ND	ND	ND	ND	↓	-	ND	ND	ND	ND
Ido2-Tot	-	-	-	↑	-	-	↓		↑ ·	↓	↑ ·	↑
Tdo2-FL	-	↑	-	↑	-	-	↑	↑	-	↑	-	↑
Tdo2-v1	-	-	-	-	-	-	↑	↑	-	↑	↑ ·	↓
Tdo2-v2	-	-	-	-	-	-	↑	↑	-	↑	↑ ·	↓
Tdo2-Tot	-	-	-	↑	-	-	↑	↑	-	↑	-	-

ND = not detected. · **Dex by LPS treatment interaction**

Downstream Kynurenine Pathway enzyme regulation by LPS, DEX and LPS+DEX

Kyn production is limited by the DOs, but Kyn is then metabolized into immune- and neuro-modulatory kynurenines associated with various pathologies. Kynurenine aminotransferase 2 (Kat2) converts Kyn into KynA, whereas kynurenine 3-monooxygenase (Kmo), kynureninase (Kynu) and 3-hydroxyanthranilate 3,4-dioxygenase (Haao) function to convert Kyn to QuinA. These downstream enzymes are expressed with tissue- and glia-specificity; we report their regulation by LPS and DEX.

Kat2 expression is largely unchanged albeit slightly induced in the liver by DEX (Figure 16A). Kat2 expression is ~10-fold higher (3.5 lower C_t) in astrocytes compared to microglia, reflecting the ability of astrocytes to produce KynA in the brain [112]. Kat2 is also highly expressed in liver supporting a well-developed ability for hepatic KynA production [192], [193].

Kmo, Kynu and Haao (Figure 16B-D) expression were not increased in brain or glia, but brain Haao is decreased 5 h after LPS treatment. Surprisingly, microglial expression of all three enzymes is diminished by LPS and DEX. Haao expression by astrocytes is also diminished by DEX. Microglial expression of these three enzymes was greater than that of astrocytes, reflecting microglial ability to generate QuinA. Kmo is induced by LPS in lung but unchanged by LPS or DEX in spleen. Kmo, Kynu and Haao are best expressed by the liver compared to the other 3 tissues; this reflects the well-accepted hepatic ability to generate QuinA and then NAD^+ from Kyn. In liver, LPS increases Kmo, but both LPS and DEX decrease Kynu and Haao expression; since Kmo acts upstream of Kynu its increase is offset by lowered Kynu. Although not considered rate-limiting, these changes suggest a slight diminution in *Kynurenine Pathway* flux towards QuinA within 5 h of acute inflammatory and stressful conditions.

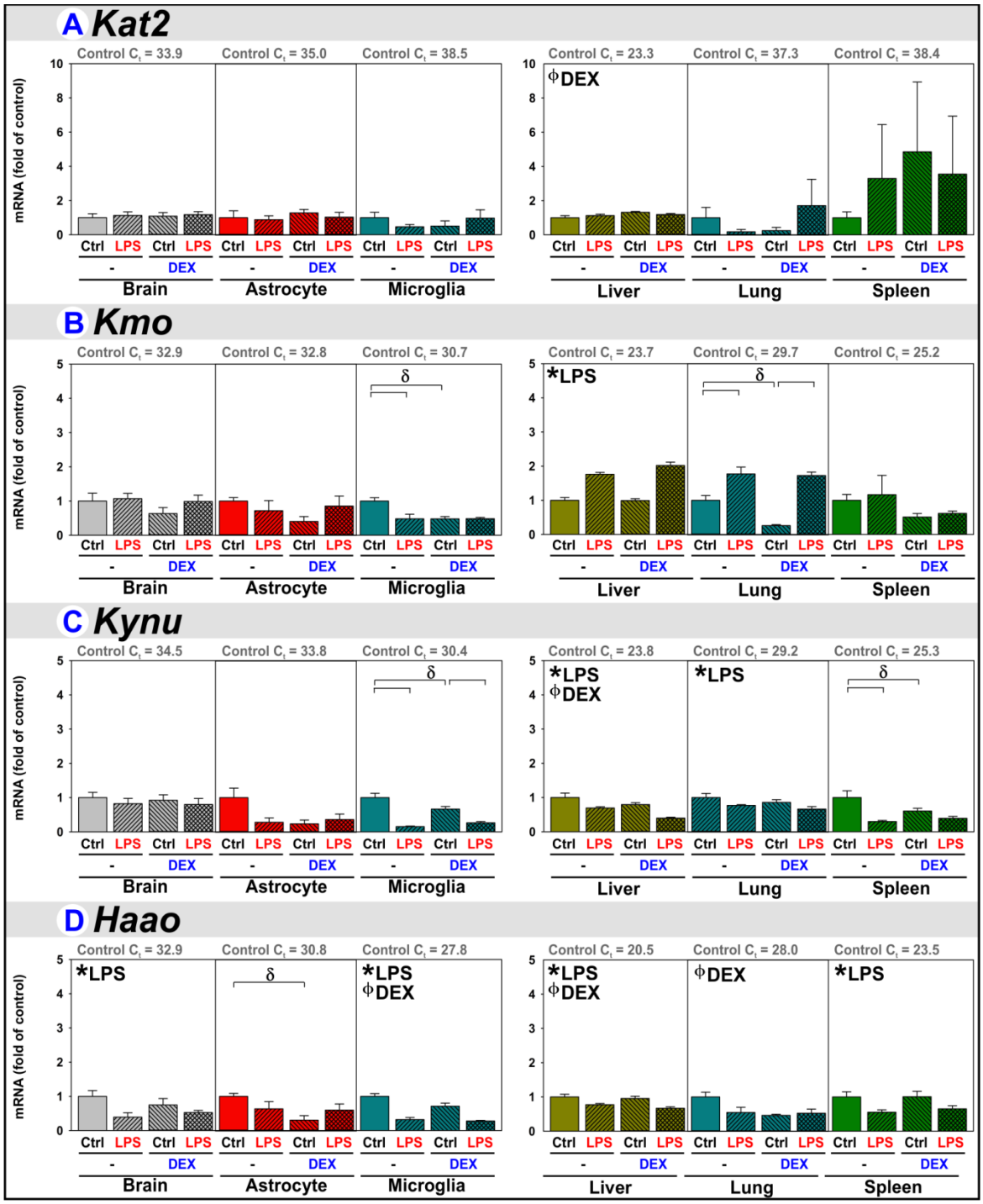

Figure 16: Downstream *Kynurenine Pathway* enzyme regulation by LPS, DEX and LPS+DEX. Brain, astrocytes, microglia, liver, lung and spleen were obtained from mice 5 h after i.p. treatment with saline (Control), LPS, DEX or LPS+DEX to assess the relative changes in the mRNA expression of **(A)** Kat2, **(B)** Kmo, **(C)** Kynu and **(D)** Haao. * $p < 0.05$ main effect of LPS. $^{\Phi}$ $p < 0.05$ main effect of DEX. $^{\delta}$ $p < 0.05$ *post hoc* mean separation in the presence of a significant LPS x DEX interaction.

3.5 Discussion

Immunogenic and psychological stress responses result in DO-dependent changes in immunophysiology [100], [101], [194]–[196], behavior [56], [71], [142] and cognition [67], [197], [198]. Changes in cytokine levels [171] and HPA axis function [199], [200] also accompany psychiatric conditions linked to DO activity [109], [110]. Although inflammatory mediators can affect HPA axis function and vice versa [25]–[27], [201], only recently have we began defining how inflammation and stress interact to control DO expression. The DOs are regulated with remarkable specificity, illustrated by the intricate interactions between inflammatory mediators and glucocorticoids on DO expression found *ex vivo* using organotypic hippocampal slice cultures (OHSCs) [57], [126], [190]. We extend those findings by describing interacting effects of pro-inflammatory (LPS) and anti-inflammatory (DEX) treatments on *Kynurenine Pathway* activity and DO expression *in vivo*.

DEX is anti-inflammatory in peripheral tissues when co-administered with LPS

Fkbp5 induction was used as an index of glucocorticoid receptor (GR) activation [133], [134]. LPS-induced Fkbp5 (Figure 11A) reflects inflammation-induced HPA-axis activation [120], [188], [202]. DEX had greater effects on Fkbp5 than LPS reflecting greater GR activation by the GR agonist [203], [204]. LPS also induces a pro-inflammatory response [33], [77], [126], [158], [202], [205]–[207], confirmed here by increases in both brain and peripheral TNFα expression (Figure 11C). DEX acted as an anti-inflammatory agent, blocking Ifnγ and Tnfα induction in peripheral tissues (Figure 11B-C). This effect was tissue-specific as DEX did not suppress brain Tnfα.

Together, these data indicate that DEX is active in the brain, but has a stronger anti-inflammatory action in the periphery. Changes in DO expression will be affected directly by

cytokines induced by LPS and by GR activation by DEX, in both the brain and periphery. Alternatively, DEX will alter DO expression by limiting the peripheral, but not central, inflammatory response.

Kyn levels increase in brain following LPS and in plasma following DEX

Changes in Kyn demonstrate functional alterations in Trp metabolism along the *Kynurenine Pathway*. Tdo2 appears to limit, not drive, blood Kyn as $Tdo2^{KO}$ mice have very high levels of circulating Kyn [208], [209]. $Ido2^{KO}$ mice have unchanged blood Kyn [100], but $Ido1^{KO}$ mice have diminished Kyn levels [85], [86], [95]. Thus, it is dogmatically accepted that elevated circulating Kyn is Ido1-dependent. Here, DEX increased plasma Kyn (Figure 12A) without changes in peripheral Ido1 expression. However, DEX increased spleen Ido2 as well as liver, lung and spleen Tdo2 expression indicating that even transient glucocorticoid release associated with HPA axis activation would be sufficient to increase circulating Kyn, albeit the source of Kyn production remains undefined.

Kyn crosses the blood-brain barrier [78], allowing peripherally administered Kyn to elevate brain Kyn [67]. Thus, plasma and brain Kyn would expectedly increase together. However, DEX increased plasma but not brain Kyn (Figure 12). By contrast, acute stress increases both plasma and brain Kyn [36], [37], [86]. The difference is that stress increases cytokine expression [86], DEX does not (Figure 11B-C). Thus, in the context of psychological stress, elevated brain Kyn could result from inflammation-induced local Kyn production.

Both plasma [53], [75], [158], [210] and brain [33], [53], [210], [211] Kyn levels are elevated when assessed 24 h after LPS administration suggesting coupling of plasma and brain Kyn. However, when LPS is administered directly into the brain, Kyn is only increased in brain and not plasma, demonstrating that the brain is fully equipped for local Kyn production [212].

Five hours after LPS treatment, brain (Figure 12B, [213]), but not plasma (Figure 12A), Kyn levels are elevated. Since plasma Kyn is not increased, an elevation in brain Kyn reflects central Kyn production. This action is likely mediated by the striking ability of LPS to increase Ido1 and Ido2 expression within both astrocytes and microglia. Together, these data demonstrate that DEX increases peripheral Kyn levels and LPS-induced inflammatory responses can independently increase central Kyn production.

LPS and DEX differentially regulate Ido1 expression in brain, glia and peripheral tissues

Inflammation and stress induce depression-like behaviors associated with Ido1 induction; such as helplessness/despair [33], [66]–[72], anhedonia [68], [71], [72] and cognitive dysfunction [67], [72]. Importantly, increased brain Ido1-FL [33], [67], [68] and Ido1-Tot [69], [71] expression are associated with these depression-like behaviors. Despite the ability of stress to enhance LPS-driven depression-like behavior [214], interactions between inflammation- and glucocorticoid-dependent Ido1 expression are poorly defined.

Ido1-FL is poorly expressed in naïve mouse brains, astrocytes and microglia (Figure 13A, see C_t values). However, central Ido1-FL is robustly induced in brain by immune activation via treatments with complete Freund's adjuvant [215], BCG [35], [142], pI:C [126] and LPS (Figure 13A, [33], [67], [77], [120], [126], [158], [189], [216], [217]). Notably, our new data demonstrate that LPS induces Ido1-FL expression by astrocytes at least as well as in microglia. Current dogma suggests that inflammation increases microglial Ido1 with resultant behavioral changes [32], [218]. Based on the current findings, astrocyte involvement in inflammation-dependent behaviors need re-evaluation [54].

Our data clearly reveal that Ido1-v1 is the major isoform expressed in naïve brain [57], [86], indicating that Ido1-v1 is the only isoform available to generate Ido1 activity in the brain. Herein we demonstrate that Ido1-v1 is induced by LPS and DEX within astrocytes (Figure 13B). Also, Ido1-v1 (not Ido1-FL) was induced by stress within astrocytes, not microglia [86], indicating that Ido1 expression by astrocytes is glucocorticoid-sensitive. Reports also demonstrate that inflammation [53] and stress [37], [125], [219] increase brain KynA, consistent with functional changes in the brain mediated by astrocytes.

Ido1 isoforms are differentially regulated in peripheral tissues (summarized in Table 2). Ido1-FL is increased by LPS and diminished by DEX in liver, lung and spleen, demonstrating a prototypical inflammatory/anti-inflammatory GR-mediated response. Conversely, Ido1-v1 is diminished by LPS in spleen and unresponsive to DEX. Together, this suggests some peripheral cell-types respond differently to inflammation and stress, but defining the significance of these unique changes in Ido1 regulation requires additional work. Clearly, the intricacies of Ido1 regulation may be overlooked with a single assay. Likely cells from each tissue respond with distinctive DO isoform profiles. Differences in Ido1 regulation will result in cell-specific changes in physiology (by generating intracellular kynurenines) and/or changes in the physiology of surrounding cells (by releasing the same kynurenines).

We recently reported that DEX potentiated cytokine-induced Ido1-FL expression by OHSCs [57]. Since i.p. LPS does not appreciably access the brain, central Ido1-FL induction by inflammatory stimuli is accepted to be mediated by cytokines [71], [75], [123], [220], especially via IFNγ and TNFα [142]. Thus, DEX might be expected to elevate LPS-induced Ido1-FL *in vivo*. However, brain, astrocyte and microglial LPS-induced Ido1-FL are not increased by DEX (Figure 13A). This discrepancy is easily explained. *Ex vivo*, the cytokine concentration is set by the

investigator and DEX potentiates Ido1-FL expression [57]. However, DEX blocked peripheral cytokine expression, resulting in less potential for synergistic induction of Ido1 in the periphery. In the brain, DEX did not attenuate LPS-induced TNFα expression so expression of Ido1-FL remains elevated. This has direct clinical implications. Depressed patients often present with mildly elevated cytokine levels [171] and/or elevated glucocorticoids [199], [200]. Activation of the *Kynurenine Pathway* may be enhanced by their combination. Thus, an underappreciated interaction between low-grade inflammation and elevated glucocorticoids may drive central DO-dependent depression.

LPS and DEX regulate Ido2 transcripts in a manner distinct from Ido1

Four laboratories independently reported the existence of Ido2 [79], [80], [221], [222]. Like Ido1, Ido2 also mediates and controls Trp metabolism to Kyn [99]. Despite performing the same enzymatic role, Ido1 and Ido2 have non-redundant functions in rheumatoid arthritis [83], [101]. This difference may result from differential regulation and/or cell-specific expression profiles as reported for liver, kidney and epididymis [80]. Also, myeloid- and plasmacytoid-dendritic cells both express Ido2, but Ido1 was detected only in myeloid-dendritic cells [99]. Thus, although sharing enzymatic function, Ido1 and Ido2 do not necessarily co-localize to the same cell types and thus can be differentially regulated with unique functional consequences.

LPS slightly increased hippocampal [120], [158] and frontal cortex [223] Ido2-Tot expression, although neither LPS nor DEX affected brain Ido2-Tot in the current study (Figure 14J). However with isoform-specific assays, we found that LPS increased Ido2-v1, Ido2-v3 and Ido2-v6 in hippocampus [126] and several Ido2 isoforms were elevated by IFNγ in OHSCs [57], [126]. Those data demonstrate isoform-specific Ido2 regulation suggesting cell-type-specific responses. Indeed, Ido2-v1 is not increased by LPS or DEX in whole brain, but LPS induced Ido2-

v1 expression in astrocytes and microglia (Figure 14A). Astrocyte Ido2-v6 expression was increased by both LPS and DEX (Figure 14D). However, the major cell type that expresses Ido2-v1 and Ido2-v6 in the brain is neither astrocytes nor microglia (Control C_t of glia >> brain). Since expression of these isoforms in brain is largely unaffected by LPS or DEX, another non-responsive cell-type(s) must express these Ido2 isoforms. Thus, changes within specific cell populations are not necessarily reflected by analysis of their resident tissue.

Of the 9 Ido2 isoforms, 8 were detected within the liver. Ido2-v1 and Ido2-v6 were increased by LPS whereas hepatic expression decreased for Ido2-FL, Ido2-v2, Ido2-v3, Ido2-v4, Ido2-v7 and Ido2-v8 (reflected by decreased Ido2-Tot). The decrease in hepatic Ido2-Tot expression by LPS (Figure 14J) is consistent with reduced hepatic Ido2 in malaria infected mice [80]. Stress also decreased hepatic expression of Ido2-FL, Ido2-v3 and Ido2-v4 [86], but DEX alone did not regulate hepatic Ido2 (Figure 14) suggesting that stress-induced down-regulation of hepatic Ido2 is not mediated by glucocorticoids, but by inflammation [86]. In the lung, Ido2-v2 is uniquely stable while other Ido2 isoforms follow an expected inflammatory induction by LPS and anti-inflammatory reduction by DEX. Spleen does not follow the same pattern. Ido2 isoforms are induced by LPS and either further induced or not changed by DEX. A mechanism explaining elevation of two hepatic Ido2 isoforms and decreases in 8 other isoforms and differential expression of isoforms in lung and spleen awaits further investigation. Likely, these changes reflect cell-type specific responses. Also, the expression profiles of Ido2 demonstrate unique patterns of regulation by LPS and DEX when compared to Ido1, consistent with previous reports and supporting non-redundant physiology.

DEX upregulates Tdo2-FL while inflammation regulates Tdo2-v1 and Tdo2-v2

The first papers confirming DO isoform existence reported high Tdo2-v1 and Tdo2-v2 in mouse liver compared to brain [55]; results now extended to include Tdo2-FL (Figure 15A, [57], [86]). Within the liver, Tdo2 activity is glucocorticoid-dependent [63]–[65], [224], in line with DEX elevating Tdo2 expression (Figure 15). Tdo2-FL was also elevated in brain, astrocytes, liver, lung and spleen (but not microglia) by DEX; suggesting that Tdo2-FL is a glucocorticoid-sensitive Tdo2 isoform, albeit with cell-type specificity.

Tdo2-v1/v2 expression by brain is unresponsive to LPS or DEX (Figure 15B-C). However, LPS (liver, spleen) and DEX (liver, lung) increase their expression in the periphery. This suggests that peripheral Tdo2-v1/v2 expression is sensitive to both pro-inflammatory mediators and glucocorticoids, but in a tissue-specific manner. Thus, inflammation- and glucocorticoid-dependent signals drive peripheral Tdo2-v1/v2 expression, whereas glucocorticoid-receptor-mediated signals drive peripheral and brain Tdo2-FL.

Downstream Kynurenine Pathway enzyme regulation by LPS, DEX and LPS+DEX

While the DOs are rate-limiting, downstream enzymes are expressed with cellular specificity and define which downstream Kyn's are produced [32], [54]. These Kyn's are categorized as neuroprotective (KynA), immunosuppressive (Kyn and KynA), neurotoxic (QuinA) and nicotinamide/NAD^+ precursor (QuinA) [32], [54]. Some of the actions of the Kyn's are mediated extracellularly by transmembrane receptors [42], [46], requiring cellular release. Other actions are mediated by intracellular receptors [97], [225]. The latter case does not require release into the extracellular fluid and as such changes in the *Kynurenine Pathway* can affect the physiology of only that cell, making physiological relevance of tissue metabolite levels difficult to interpret. The best example, extracellular QuinA is neurotoxic via the NMDA-R [226],

but intracellular QuinA is used to generate NAD^+ [227] and thus the *Kynurenine Pathway* is needed for neuroprotection [228]. Thus, changes in downstream enzyme expression could shift the relative production of these Kyn's.

Kat2 facilitates Kyn metabolism to KynA. Kat2 is preferentially expressed by astrocytes (Figure 16A) [137] and neurons [156]. Stress increases brain KynA [37], [119], [125]. However, these changes were not associated with changes in Kat2 expression (Figure 16A), [86], [119], [125]. Similarly, LPS increases central KynA without changes in Kat2 [53]. These findings demonstrate that KynA levels change independently of Kat2, confirming that KynA generation is dependent on Kat2 substrate availability (i.e. increased astrocyte DO-dependent Kyn production).

Kmo, Kynu and Haao facilitate QuinA generation from Kyn. In brain, these enzymes are predominantly expressed by microglia (Figure 16B-D), [32], [54]. In the current study, LPS and/or DEX ~halved Kynu and Haao expression in microglia, liver, lung and spleen, albeit doubling Kmo in liver and lung (Figure 16B-D). These finding agree with other work showing only minor changes in these enzymes by inflammation [158] and stress [86], [119] compared to Ido1 and Ido2 induction (Figure 13 and Figure 14). These and other data suggest that changes in plasma or sera levels of Kyn's are dependent on changes in expression of the rate-limiting DOs [52], [210], [229]–[231]. Even so, enzymes downstream of the DOs are important therapeutic targets [50], [53]. Thus, examining defects-in or therapeutic manipulation-of downstream *Kynurenine Pathway* activity is an active area of preclinical research.

Conclusions

Here we show that Ido1, Ido2 and Tdo2 are uniquely regulated by LPS and DEX with elaborate cell-, tissue- and isoform-specificity. We identify DO isoforms uniquely adapted to

independently change in response to inflammation and glucocorticoids. Although only the first step towards defining the biological significance of their expression, our data begin to unravel the complex interactions between inflammation and stress regarding activation of *Kynurenine Pathway* enzymes under these often comorbid conditions. Importantly, the effects of inflammation and stress require different assays to identify treatment-specific changes in DO isoform expression. For instance, we show that both astrocytes and microglia respond to LPS by increasing Ido1-FL expression, but DEX increases Ido1-v1 expression by both cell-types. The expression of Ido1 was not attenuated by DEX in brain as was observed in peripheral tissues, demonstrating that the brain displays a differential response in the context of an inflammatory challenge coupled with elevated glucocorticoids. Taken together, our current findings provide novel evidence of a brain-specific interaction between inflammation and stress which may define a unique mechanism pivotal to the depression susceptibility of individuals affected by chronic inflammatory disease.

CHAPTER 4: Ido1 expressed by myeloid-derived cells contributes to brain *Kynurenine Pathway* activity and inflammation-induced anhedonia [3]

4.1 Abstract

Depression is associated with central neuro-inflammation and symptoms of depression manifest following central or peripheral pro-inflammatory stimuli. In humans and rodents, respectively, symptoms of depression and depression-like behavior have been associated with the cytokine-inducible enzyme indoleamine 2,3-dioxygenase (Ido)1. While global Ido1 knockout mice are protected from anhedonia following an immune challenge with lipopolysaccharide (LPS), the cellular sources of Ido1 underpinning this effect remain unknown. To address this issue, we bred mice with lox-P gene inserts flanking Ido1 to mice expressing Cre-recombinase in myeloid-derived cells including brain microglia (M-Cre, *Lyz2* promoter) or neurons (N-Cre, *Camk2a* promoter) generating cell-type specific Ido1 knockdown models. We hypothesized both myeloid- and neuron-derived Ido1 could contribute to anhedonia following a peripheral injection with LPS. Loss of myeloid Ido1 ($Ido1^{f}$xM-Cre mice) attenuated the LPS-induced anhedonic response (assessed as sucrose preference), whereas loss of neuronal Ido1 ($Ido1^{f}$xN-Cre mice) had no effect on LPS-induced anhedonia. These behavioral responses paralleled a decreased kynurenine to tryptophan (Kyn/Trp) ratio in the brains of $Ido1^{f}$xM-Cre mice but not in the brains of $Ido1^{f}$xN-Cre mice. Plasma Kyn/Trp was not affected in either knockdown model. These results indicate that microglia-derived (and not neuron-derived) Ido1 contributes to the anhedonic response following a peripheral inflammatory challenge. LPS induced brain Ifnγ and

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[3] Edited from original manuscript *in prep* with headings, figures, and tables renumbered:
Dostal CR, *et al.* Ido1 expressed by myeloid-derived cells contributes to brain *Kynurenine Pathway* activity and inflammation-induced anhedonia. *in prep*

Tnfα mRNA expression to a similar degree in both Ido1xM-Cre and Ido1xN-Cre mice relative to their respective controls, indicating that the protective effect was not associated with diminished neuroinflammation. LPS-induced upregulation of Ido1-v1 (the main Ido1 transcript found in the mouse brain), but not Ido1-FL, was absent in both $Ido1^{f}$xM-Cre and $Ido1^{f}$xN-Cre mice. Collectively, these data suggest that Ido1 within neurons and microglia play distinctive roles in animal behavior.

4.2 Introduction

Depression is the leading cause of global disability affecting over 350 million people worldwide [232], with an annual economic burden estimated at $70 billion and rising [233]. Primary depressions are rare as over 70% of cases present with one or more comorbidities [108]. Patients with conditions that chronically activate the immune system are especially vulnerable to depression, even when they have no prior history of mental disorders [29], [234]. The *Monoamine Theory of Depression* hypothesized that major depressive disorders results from reduced monoaminergic (*e.g*. dopaminergic, adrenergic, serotonergic) signaling; however, evidence-based pharmacotherapy fails to fully substantiate this claim [28], [40]. In fact, anti-depressants have marginal efficacy and unfavorable side-effects [235]. More recently, evidence from human and animal studies supporting a link between inflammation, depression and *Kynurenine Pathway* metabolism has laid the groundwork for proposing the new *Kynurenine Theory of Depression* which holds that an imbalance in central levels of kynurenine (Kyn) metabolites mediate or precipitate symptoms of depression [32].

The observation that depression symptomology is associated with elevated *Kynurenine Pathway* metabolite levels in patients receiving cytokine treatments for cancer and hepatitis C [236], [237] has resulted in large-scale investigations into the immune system's role in

depression [28], [29], [31], [34], [40], [73], [74], [76], [77], [159], [234], [236]–[241]. Kynurenine (Kyn) is generated from tryptophan (Trp) by three rate-limiting indoleamine/tryptophan 2,3-dioxygenases (DOs: Ido1, Ido2 and Tdo2) [54]. These DOs and their various isoforms are regulated with incredible specificity by inflammation [57], [126], [190], [242] and stress [86].

Animal models have been used to show that systemic administration of Kyn to mice dosage-dependently elicits depressive-like behaviors [33]. While Kyn is transported across the blood-brain barrier[78], Kyn itself is not widely considered as neuro-modulatory. Kyn must be metabolized into its downstream neuro-active metabolites, the downstream kynurenines (Kyns) [54]. Importantly, the enzymatic machinery downstream of the DOs are cell-specific, resulting in specialized cellular generation of Kyns [54]. Specifically, microglia release quinolinic acid (QuinA) [124] while neurons produce more kynurenic acid (KynA) and picolinic acid (PicA) [243] (Figure 17). Several publications support the current dogma which holds that inflammation induces Ido1-dependent QuinA secretion by microglia. The neuro-toxic QuinA then mediates Ido1-dependent depression-like behaviors [67]. However, to our knowledge this is the first report to directly test this hypothesis using Cre-Lox technology to generate cell-type specific Ido1-knockdown mice.

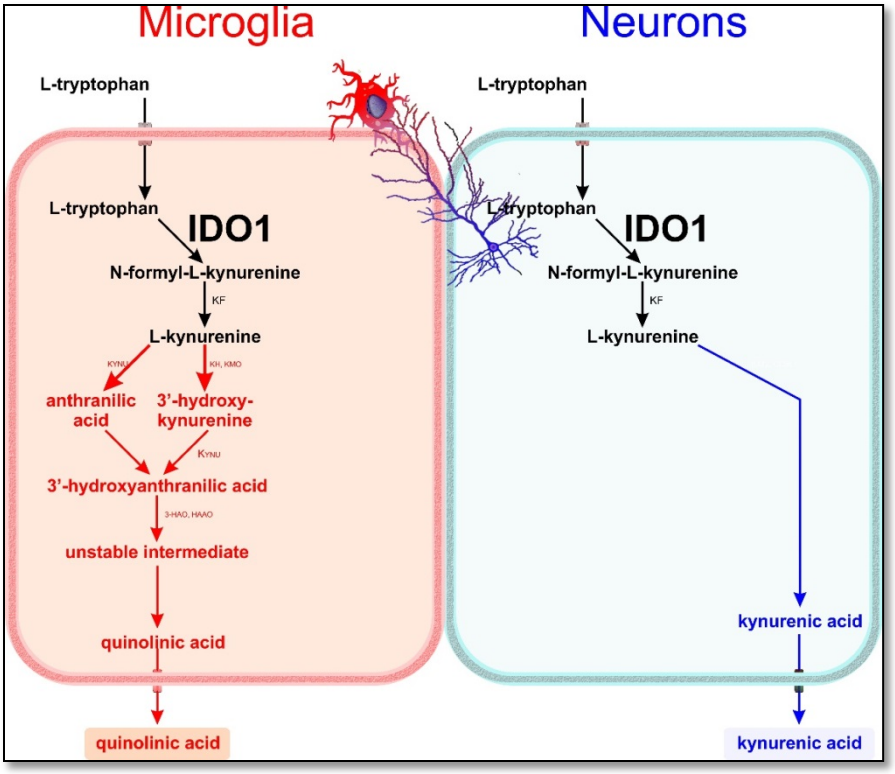

Figure 17. Increased Indoleamine 2,3-dioxygenase (IDO) 1 activity in microglia and neurons result in the production of distinct neuroactive metabolites. Ido1 expressed in microglia is rate-limiting for the production of quinolinic acid, while Ido1 expressed in neurons is rate-limiting for the production of kynurenine acid. [32], [124], [243]

4.3 Methods

Animals

Ido1 transgenic 'floxed' mice (Ido1^{f}) were generated by inGenious Targeting Laboratory (Stony Brook, NY). Exons 3-5 were flanked by loxP sites enabling Cre-recombinase excision of the three exons. Comparison of the excision site to the commonly used Ido1 knockout mouse is shown in Figure 18. Cell-type specific knockdown mice were generated by breeding Ido1^{f} mice to mice expressing Cre-recombinase in either myeloid-derived [244], [245] cells (M-Cre: stock # 004781, B6.129P2-Lyz2$^{tm1(cre)Ifo}$/J, Jackson Laboratories, Bar Harbor, MN) or in neurons [246], [247] (N-Cre: stock # 012362, B6;129S6-Tg(Camk2a-cre/ERT2)1Aibs/J, Bar Harbor, MN). All mice are on a C57BL/6J background and floxed mice were bred to homozygosity such that both Ido1

alleles could be excised by Cre-recombinase. Cre-mediated recombination is constitutive in M-Cre mice, but recombination was induced in N-Cre strains with 1-week *ad lib* access to tamoxifen-supplemented chow (TD.130857, Harlan Laboratories, Madison, WI).

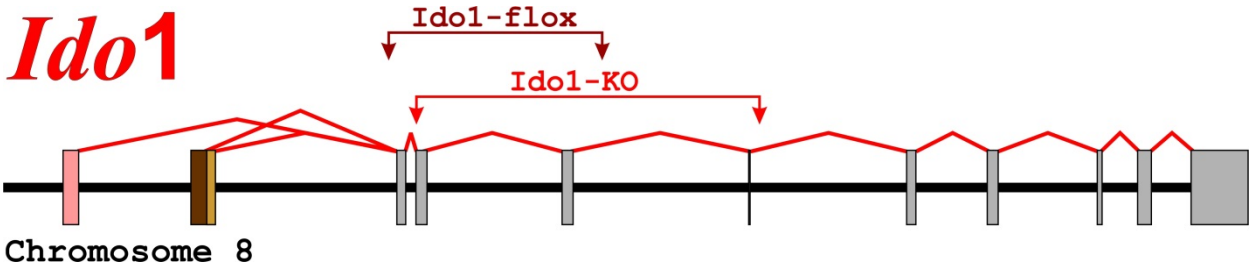

Figure 18: Illustration of Ido1 excision sites. The Ido1-flox mouse used in this study has exons 3-5 flanked by loxP for Cre-mediated cell-specific excision. For comparison, the exons removed (4-6) from the commonly used Ido1 knockout mouse (Ido1-KO) are also illustrated.

Male mice were housed on reversed 12 h light-dark cycle with *ad lib* access to chow and water. Mice were individually housed at least 2 weeks and handled for 5 consecutive days prior to experiments. Mice were 12 to 15 weeks of age at the time of study. Animal procedures were approved by the Institutional Animal Care and Use Committee and performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council).

Genotyping and verifying Cre-lox mediated recombination.

After mice were sacrificed and intracardially perfused with 4°C saline, brain and liver was harvested and processed for DNA. Mice genotype was verified by PCR using the following primers:

Cre forward:	AATGCTTCTGTCCGTTTGCCGGT
Cre reverse:	CCAGGCTAAGTGCCTTCTCTACA
Ido1 floxed forward:	GGGCTTCCAAGTGCTGTCTATG
Ido1 Δ forward:	GCTGACAACCTTAAAGCCATCTCATTC
Ido1 reverse:	CGAGAGTGTATTTTCTGGGTGC.

Following electrophoresis through 7.5 % polyacrylamide gels and staining with ethidium bromide, the presence of Cre was confirmed in brain tissue by observation of a 518 bp band

using a LAS-4000 (Fujifilm Life Sciences, Stamford, CT). Ido1 flox status was also confirmed in brain tissue (WT allele 564 bp band, floxed allele 740 bp band), as well as Cre-mediated recombination (Δ allele 390 bp band) (Figure 19). While the Δ band was constitutively present in brains of naïve Ido1^{f}xM-Cre mice, the Δ band was only present in the brains of tamoxifen fed Ido1^{f}xN-Cre animals (data not shown). This confirms the constitutive activity of the M-Cre recombinase and the conditional tamoxifen-induced activation of the N-Cre recombinase, respectively. Moreover, the Δ band was present in livers of Ido1^{f}xM-Cre mice but not tamoxifen-fed Ido1^{f}xN-Cre mice (data not shown), illustrating tissue-specificity for the neuronal Ido1 knockdown model but the ability of M-Cre to also excise the Ido1 exons in myeloid-derived cells of a peripheral tissue.

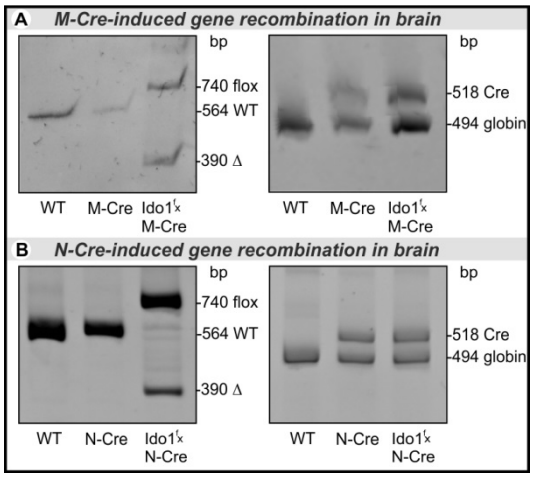

Figure 19: Cre-induced gene recombination in brain. PCR on DNA extracted from saline perfused brain tissue from wild-type (WT) mice and **(A)** M-Cre strains or **(B)** N-Cre strains verified the Ido1 WT allele (564 bp) Ido1-flox allele (740 bp) and recombinant Δ allele (390 bp) as indicated bands on the left panels. The presence of the Cre allele (518 bp) was also verified as indicated by bands on the right panels.

Sucrose Preference, Body Weight and Food Intake.

Mice were trained for 4-5 d with access to a bottle containing water and an identical bottle containing 1% sucrose. Each morning, both bottles were weighted and change in weight was calculated for both the water and sucrose bottles. The 24 h sucrose preference was

calculated as a percentage: [(gram change in sucrose)/(gram change in sucrose + gram change in water)] x 100. Training was complete when all mice had established a greater than 85% preference for the sucrose solution. Mice were then injected immediately prior to onset of the dark cycle with either saline (controls: Ctrl) or saline to deliver 0.83 mg/kg body weight of lipopolysaccharide (LPS serotype O127:B8, Sigma). Sucrose preference, body weights and chow consumption were recorder for the next 4 days after treatment.

Table 3: Effect of LPS on body weight and food intake.

	M-Cre		Ido1^{fx}M-Cre	
	Ctrl	LPS	Ctrl	LPS
Δ Body Wt. (g)	0.4 ± 0.1	-1.1 ± 0.1 *	0.8 ± 0.2	-1.1 ± 0.2 *
Food Intake (g)	0.9 ± 0.1	0.1 ± 0.1 *	1.0 ± 0.2	0.0 ± 0.2 *
	N-Cre		Ido1^{fx}N-Cre	
	Ctrl	LPS	Ctrl	LPS
Δ Body Wt. (g)	0.0 ± 0.1	-1.6 ± 0.1 *	0.0 ± 0.2	-1.6 ± 0.2 *
Food Intake (g)	1.5 ± 0.1	0.2 ± 0.1 *	1.6 ± 0.1	0.2 ± 0.1 *

* $p < 0.05$ for the effect of LPS

Quantitative polymerase chain reaction (qPCR)

Ctrl and LPS-treated mice were euthanized using CO_2 5 h after treatment. Following intracardial perfusion with ice-cold saline, brains were removed, homogenized, suspended in TRIzol and stored at -80 °C until processed for gene expression. Materials and methods for RNA extraction, reverse-transcription and qPCR were recently described [86]. Expression of each test gene is normalized to Gapdh using the $2^{-\Delta\Delta Ct}$ method [131]. Gene expression for the Ctrl groups is set to 1.0 and other groups are expressed as fold of Ctrl. Ido1 gene structure, mRNA transcripts and probe-based assays (IDT, Coralville, Iowa) quantifying each Ido1 transcript were recently described [86]. Because of low expression, some transcripts are 'not detectable' in all samples (i.e. C_t values 'undetermined') preventing calculation of relative gene expression levels. For analysis a C_t value of 40.0 is assigned when this occurs. Treatment efficacy during the 5 h

post-treatment period was confirmed by a reduction of body weight and food intake by LPS (Table 3). These changes were unaffected by Ido1 status.

High performance liquid chromatography (HPLC) for Kyn.

A separate cohort of mice were treated with saline (Ctrl) or LPS and sacrificed 24 h after treatment. Blood was collected from the inferior vena cava immediately prior to intracardiac perfusion with ice-cold saline. After perfusion, brains were harvested, snap frozen in liquid N_2 and then stored at -80 °C. To measure Trp and Kyn concentrations, the brain and plasma samples were processed for HPLC and quantified using electrochemical detection as recently described [86], [95], [132], [242].

Statistics

Data are presented as means ± SEM representing 4-8 mice per group. Two-way repeated measures ANOVA was used when determining significant treatment effects at different time points. Two-way ANOVA was used when determining significant effects of LPS and genotype at a single time point. Significance was set at $p \leq 0.05$. Significant main effects of LPS or genotype are annotated with * or $^{\phi}$, respectively. Data annotated with $^{\delta}$ represent significant effects by *post hoc* analysis using the Holm-Šídák method when a significant LPS x genotype interaction occurred. Plotting and statistical analysis were completed using SigmaPlot 13.0.

4.4 Results

Mice deficient for Ido1 in either myeloid-derived cells (Ido1^{f}xM-Cre) or neurons (Ido1^{f}xN-Cre) were generated to determine if Ido1 expressed in these cell-types contribute to anhedonic-like behavior induced by inflammation [68].

Myeloid-derived Ido1 contributes to symptoms of inflammation-induced anhedonia and brain Kynurenine Pathway activity and Ido1 expression.

Peripheral administration of LPS results in an acute sickness response readily measured as a loss of body weight [29], [33]. Expectedly, 24 h after treatment the LPS-treated mice experienced reduction in body weight relative to saline treated mice (Figure 20A). This effect occurred independent of genotype suggesting that the Ido1 in myeloid-derived cells does not contribute to the LPS-induced sickness response. Likewise, 5 h after LPS treatment, brain Ifnγ and Tnfα gene expression increased similarly in both M-Cre and Ido1^{f}^{x}M-Cre mice relative to their saline treated Ctrls (Figure 20C). However, LPS-treated mice demonstrated reduced sucrose preference in the 24 h following treatment with a significant treatment x time interaction such that LPS-induced anhedonia was attenuated in Ido1^{f}^{x}M-Cre mice relative to M-Cre mice (Figure 20B). Inflammation-induced anhedonia was resolved in both M-Cre and Ido1^{f}^{x}M-Cre mice with no treatment-effects of LPS treatment by 48 h after treatment.

The plasma Kyn/Trp ratio was increased in LPS treated mice independently of genotype, demonstrating that this well-documented effect occurring 24 h after treatment is independent of Ido1 derived from cells of myeloid lineage (e.g. monocytes, macrophages, neutrophils, etc.). Importantly, Ido1^{f}^{x}M-Cre mice have lower brain Kyn/Trp relative to M-Cre mice independently of LPS-treatment suggesting that myeloid-derived cells contribute to *Kynurenine Pathway* activity in the brain (Figure 20D). Moreover, 5 h after LPS treatment, brain Ido1-FL expression increased 200-fold in M-Cre mice but only 110-fold in Ido1^{f}^{x}M-Cre mice, albeit this occurred independently of genotype (Figure 20E). Thus, LPS-induced Ido1-FL expression was numerically but non-significantly lower in brains of LPS-treated Ido1^{f}^{x}M-Cre mice relative to LPS-treated M-Cre mice. Brain Ido1-v1 expression was induced by LPS 5 h after treatment in M-Cre control

mice but was not LPS-inducible in Ido1^{f}^{x}M-Cre mice (Figure 20E). Brain Ido1-v2 expression was not significantly induced by LPS 5 h after treatment; however, brain Ido1-v2 expression was ~3-fold greater in Ido1^{f}^{x}M-Cre versus M-Cre mice suggesting some compensatory upregulation of Ido1-v2 expression in brain by an unidentified and non-myeloid-derived cell-type(s).

These data demonstrate that myeloid-derived Ido1 specifically contributes to inflammation-induced anhedonic-like behavior (Figure 20B), as well as brain (but not plasma) *Kynurenine Pathway* activity (Figure 20D). Importantly, unlike global Ido1^{KO} mice [68], these animals are not completely protected from the reduced sucrose preference initiated by i.p. LPS suggesting that Ido1 activity in other cells within the brain also contribute to Ido1-depenent anhedonic behavior induced by inflammation.

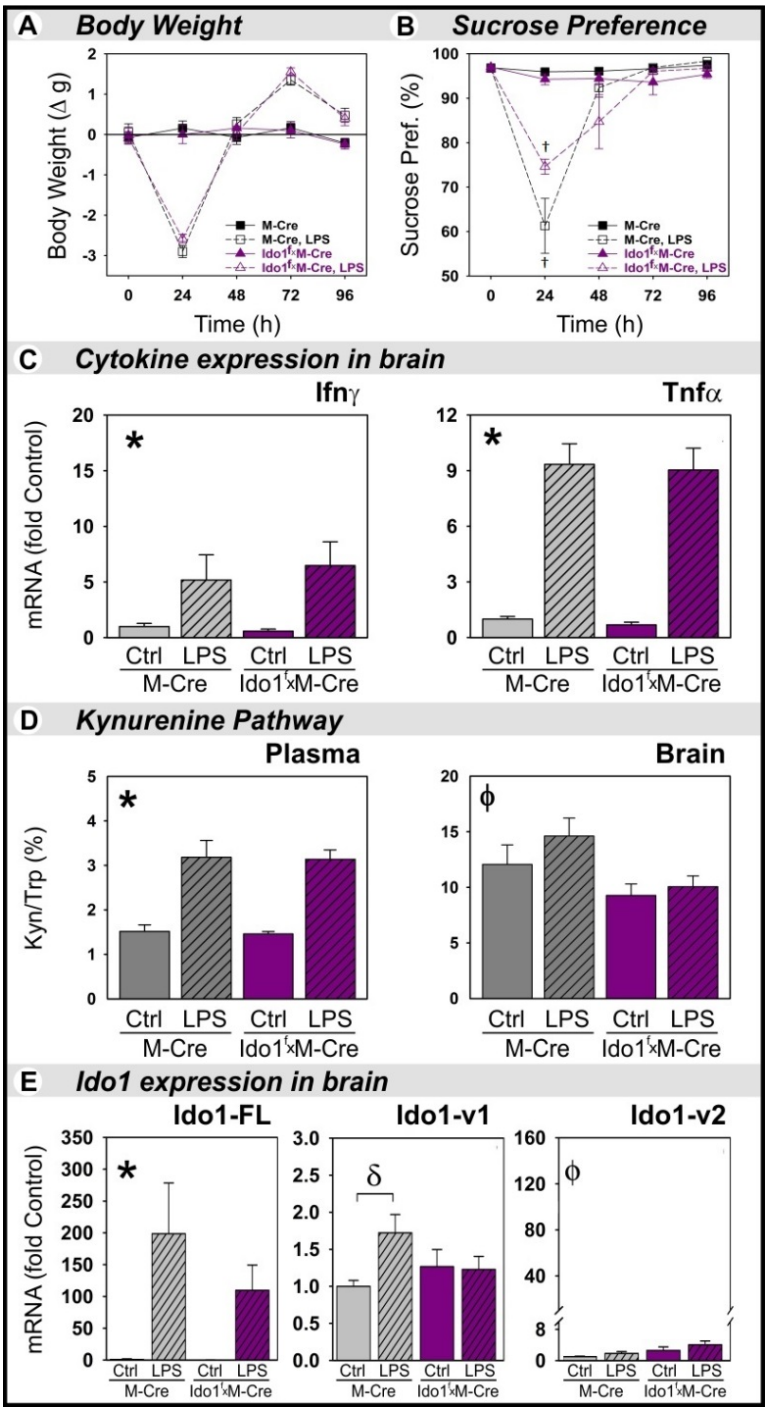

Figure 20. Myeloid-derived Ido1 contributes to symptoms of inflammation-induced anhedonia and brain Kynurenine Pathway activity and Ido1 expression. Mice deficient in myeloid-derived Ido1 (Ido1fxM-Cre) and their genetic controls (M-Cre) were treated i.p. with saline (Ctrl) or saline containing LPS and then monitored for changes in **(A)** body weight and **(B)** sucrose preference to determine differences in LPS-induced anhedonia. 5 h after Ctrl or LPS treatments, **(C)** brain mRNA expression of Ifnγ and Tnfα is measured. 24 h after treatments, the (D) plasma and brain Kyn/Trp ratio is assessed representing Kynurenine Pathway activity. 5 h after treatments, (E) brain mRNA expression of Ido1-FL, Ido1-v1, and Ido1-v2 is measured. † represents $p < 0.05$ by 2-way repeated measures ANOVA when a significant treatment x time interaction occurs. * represents $p < 0.05$ main effect of LPS, ϕ represents $p < 0.05$ main effect of mouse genotype, δ represents $p < 0.05$ when a significant LPS x genotype interaction occurs by 2-way ANOVA.

Neuron-derived Ido1 makes transcript-specific contributions to brain Ido1 gene expression.

LPS treatment resulted in reductions in both body weight (Figure 21A) and sucrose preference (Figure 21B) relative to saline treatments (Ctrl); these effects were independent of neuronal Ido1 knockdown status. Thus, unlike the mice deficient in myeloid-derived Ido1, the inflammation-induced sickness response and anhedonia-like behavior are both similar in Ido1^{fx}N-Cre mice and N-Cre mice. Likewise, 5 h after treatment the LPS-induced increases in brain Ifnγ and Tnfα gene expression were completely independent of neuronal-Ido1 knockdown status (Figure 21C). Thus, neuronal-Ido1 knockdown does not result in differences in neuro-inflammation initiated by i.p. LPS treatments. Relative to Ctrl, LPS also increased plasma and brain Kyn/Trp to a similar extent in N-Cre and Ido1^{fx}N-Cre mice when assessed 24 h after treatment (Figure 21D). This indicates that neuronal-Ido1 does not contribute to LPS-induced increases in either brain or plasma Kyn/Trp. Thus, conditional knockdown of neuronal-Ido1 does not protect mice from LPS-induced anhedonia-like behavior, nor does neuronal-Ido1 knockdown perturb the neuro-inflammatory response initiated by LPS or plasma and brain Kyn/Trp levels in naïve or LPS treated mice.

Ido1 expression was assessed in whole-brain 5 h following i.p. Ctrl or LPS injections. Ido1-FL expression was increased 320-fold in both N-Cre and Ido1^{fx}N-Cre by LPS independently of genotype (Figure 21E). Ido1-v1 expression was upregulated by LPS in N-Cre control mice; however, it was not LPS-inducible in Ido1^{fx}N-Cre mice. Ido1-v2 gene expression was 72-fold greater in Ido1^{fx}N-Cre mice relative to N-Cre control genotypes; however, Ido1-v2 was not LPS inducible in either N-Cre or Ido1^{fx}N-Cre mice. This demonstrates major compensatory upregulation of Ido1-v2 expression in mice deficient in neuron-derived Ido1.

Together, these data suggest that neuron-derived Ido1 plays no role in inflammation-induced anhedonic-like behavior. However, Ido1^{f}^{x}N-Cre mice also exhibits remarkable compensatory upregulation of Ido1-v2 when compared to N-Cre controls which likely compensates for neuronal-Ido1 knockdown within in a non-neuronal and undetermined cell-type(s). Because of this striking upregulation of Ido1-v2, the neuronal contribution of Ido1 to inflammation-induced anhedonia-like behavior remains unconfirmed.

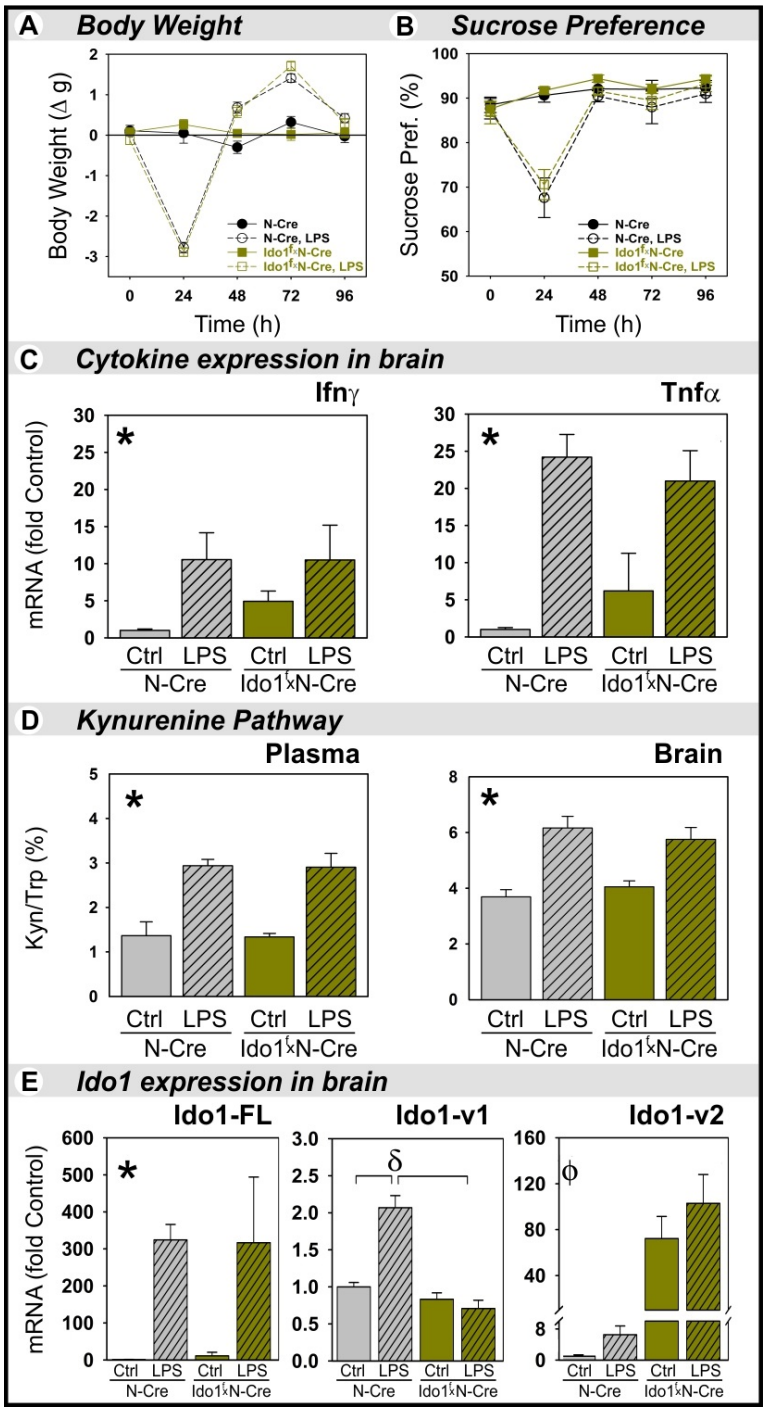

Figure 21: Neuron-derived Ido1 makes transcript-specific contributions to brain Ido1 gene expression. Mice deficient in neuron-derived Ido1 (Ido1^{f}xN-Cre) and their genetic controls (N-Cre) were treated i.p. with saline (Ctrl) or saline containing LPS and then monitored for changes in **(A)** body weight and **(B)** sucrose preference to determine differences in LPS-induced anhedonia. 5 h after Ctrl or LPS treatments, **(C)** brain mRNA expression of Ifnγ and Tnfα is measured. 24 h after treatments, the **(D)** plasma and brain Kyn/Trp ratio is assessed representing *Kynurenine Pathway* activity. 5 h after treatments, **(E)** brain mRNA expression of Ido1-FL, Ido1-v1, and Ido1-v2 is measured. * represents $p < 0.05$ main effect of LPS, $^{\phi}$ represents $p < 0.05$ main effect of mouse genotype, and $^{\delta}$ represents $p < 0.05$ when a significant LPS x genotype interaction occurs by 2-way ANOVA.

4.5 Discussion

Inflammation-induced anhedonia initiated by LPS administered into the periphery or brain is Ido1-dependent [68], [212]; however, the Ido1 expressing cell-type(s) mediating this response is undefined. Since microglia [76], [142], [189], [242] and neurons [124], [243], [248] respond to inflammation with Ido1 upregulation, we generated mice deficient in either myeloid- or neuron-derived Ido1 to distinguish contributions of these cell-types to inflammation-induced anhedonia.

Myeloid-derived Ido1 contributes to symptoms of inflammation-induced anhedonia and brain Kynurenine Pathway activity and Ido1 expression.

Mice deficient in myeloid-derived Ido1 are protected from the full spectrum of the anhedonic response induced by LPS (Figure 20B). The M-Cre system is effective at excising floxed alleles of both central (e.g. microglia) and peripheral (e.g. monocytes) myeloid-derived cells [244], [245]; however, Ido1^{f}xM-Cre mice have reduced Kyn/Trp in saline perfused brain, not plasma (Figure 20D), demonstrating a functional effect of myeloid-derived Ido1 knockdown on brain Ido1 activity. While the increases in central and peripheral *Kynurenine Pathway* activity following LPS injection are well documented [54], [242], the cellular origins driving increased Kyn/Trp have been undefined. Our data indicates that LPS increases peripheral Kyn/Trp independently of Ido1 induction by myeloid-derived cells (e.g. Macrophages, monocytes, neutrophils, etc). Clearly, central myeloid-derived cells influence brain Kyn/Trp, but these cells may include perivascular, meningeal, and choroid plexus macrophages in addition to parenchymal microglia [249]. Likewise, LPS treatment reportedly increases trafficking of myeloid-derived cells from the blood into the brain where their phenotype may be altered [250], [251]. Nonetheless, precisely determining to what extent Ido1 from each of these various

myeloid-derived cells contributes to brain *Kynurenine Pathway* activity was out of the scope of the current study.

LPS treatment induces Ido1-FL in microglia [189], [190], [242], and inflammation-induced Ido1-FL is cytokine-dependent [75], [142]. Importantly, we verified that Ido1^{f}xM-Cre and M-Cre mice have similar brain cytokine responses to LPS treatment (Figure 20C) which is consistent with reports of LPS inducing pro-inflammatory cytokines to a similar extent in brains of wild-type and global Ido1^{KO} mice [67], [212]. Brain Ido1-FL expression in LPS treated Ido1^{f}xM-Cre mice was nearly ½ of Ido1-FL expression in LPS treated M-Cre mice (Figure 20E). The reduction of Ido1-FL expression in the brain of Ido1^{f}xM-Cre mice was non-significant as microglia only represent a small fraction of brain cells; most other cell-types in the brain are expected to respond to LPS treatment with Ido1-FL potently induced [54], making the effects of myeloid-targeted Ido1 knockdown difficult assess in brain.

Total knockout strategies have demonstrated that Ido1 is necessary for LPS-induced anhedonia [68]; however, until this report the cellular-specificity of this response was undefined. These data strongly suggest that myeloid-derived cells within the brain (i.e. microglia, brain-associated macrophages) contribute to the inflammation-induced anhedonic response of mice.

Neuron-derived Ido1 makes transcript-specific contributions to brain Ido1 gene expression.

Mice deficient in neuron-derived Ido1 (Ido1^{f}xN-Cre) respond with similar LPS-induced anhedonic-like behavior as their LPS treated control (N-Cre) mice (Figure 21B). The neurons expressing Cre recombinase in this model are glutamatergic [252]. The Camk2a promoter

targets Cre recombinase expression mostly to excitatory neurons in forebrain areas, including the hippocampus and striatum [247]. While Ido1 derived from these excitatory neurons may not be driving the inflammation-induced anhedonia of mice, these excitatory neurons project to other brain regions of the mesolimbic dopamine reward system such as the nucleus accumbens and the ventral tegmental area [253]. Neuronal production of kynurenine acid (KynA) [156], [243] in these brain regions may regulate dopamine [254], [255] or GABA [256] synaptic transmission. Nevertheless, determining the extent to which Ido1 derived from other neuronal cell-types including GABAergic neurons of the nucleus accumbens or dopaminergic neurons of the ventral tegmental area was out of the scope of the current work.

As N-Cre and Ido1^{f}xN-Cre responded to LPS with similarly elevated plasma and brain Kyn/Trp (Figure 21D), the LPS-induced expression of Ido1-FL in brains of these mice was also similar (Figure 21E). Remarkably, Ido1-v2 expression is ~80-fold greater in brains of Ido1^{f}xN-Cre mice relative to N-Cre controls. The Ido1-v2 transcript is poorly expressed in the naïve mouse brain where its expression is still undetectable after LPS treatment, albeit LPS-sensitive in lung tissue [242]. Thus, the compensatory upregulation of Ido1-v2 in whole brain of Ido1^{f}xN-Cre mice is a surprising result; however, the brain-region(s) or cell-type(s) mediating this effect remains undefined.

While global Ido1KO mice are protected from LPS-induced anhedonia [68], the effects of N-Cre mediated neuronal-Ido1 knockdown on animal behavior were difficult to ascertain because of the apparent upregulation of Ido1-v2 in a non-neuronal cell-type(s) of Ido1^{f}xN-Cre mice.

Conclusions

The normal hedonic activity (sucrose preference) of naïve mice was unaffected by either myeloid- or neuronal-knockdown of Ido1. However, mice deficient in myeloid-derived Ido1 (Ido1^{f}xM-Cre) had an attenuated anhedonic response to i.p LPS. Similar proinflammatory responses were induced by LPS in both Ido1^{f}xM-Cre and Ido1^{f}xN-Cre mice relative to their genetic controls. Even so, the Kyn/Trp ratio was only lower in brains, not plasma, of Ido1^{f}xM-Cre mice, suggesting Ido1 from microglia (and not neurons) contribute to inflammation-induced anhedonia. The mRNA expression of three Ido1 isoforms was quantified in whole brain. LPS-induced Ido1-v1 upregulation was absent in both Ido1^{f}xM-Cre and Ido1^{f}xN-Cre mice, but not their respective controls. However, there was a remarkable upregulation of Ido1-v2 in brains of Ido1^{f}xN-Cre mice relative to N-Cre control brains, suggesting glial compensatory upregulation in response to neuronal Ido1 knockdown. Unlike N-Cre strains, the Ido1-FL induction by LPS in brains of M-Cre control mice was roughly double that of Ido1^{f}xM-Cre mice, signifying that Ido1-FL is not full induced due to microglia Ido1 knockdown. Together, these data demonstrate that Ido1 within neurons and microglia play distinct role in animal behavior, and that microglia-derived Ido1 likely contributes to inflammation-induced depression-like behavior.

CHAPTER 5: Conclusion

5.1 Conclusion

These data recognize a unique role for astrocytes in the DO response to acute stress without any significant effects of acute stress on the DO regulation in microglia. Moreover, we describe unique regulation patterns for microglia and (a more underappreciated role for) astrocytes and in the DO response to peripheral inflammation and exogenous glucocorticoid. Importantly, glucocorticoids fail to suppress inflammation-induced DOs with the brain and glia whereas they are effective on DOs in the periphery in a DO-specific and tissue-specific manner. Finally, possibly owing to a robust compensatory upregulation of Ido1-v2 following neuronal knockdown of Ido1, there were no discerned effects neuronal Ido1 knockdown on animal behavior. Both myeloid- and neuronal-Ido1 knockdown mice did not upregulated Ido1-v1 in response to LPS; however, their respective genetic control mice had normal Ido1-v1 responses. Lastly, these data strongly suggest microglia contribute to inflammation-induced anhedonia-like behavior in the Ido1^{f}xM-Cre mice. Together, these data highlight DO regulation by stress and inflammation in a glia- and tissue-specific manner, and cell-specific contributions of Ido1 to inflammation-induced anhedonia-like behaviors in mice.

5.2 Publications

1. Zhai, L, Dey M, Lauing KL, Gritsina G, Kaur R, Lukas RV, Nicholas MK, Rademaker AW, **Dostal CR**, M^{C}Cusker RH, Raizer JJ, Parsa AT, Bloch O, Wainwright DA. The kynurenine to tryptophan ratio as a prognostic tool for glioblastoma patients enrolling in immunotherapy. *J. Clin. Neurosci.* 22, 1964–1968 (2015).
2. Lawson MA, **Dostal CR**, Brooks AK & M^{C}Cusker RH. The Kynurenine (Kyn) Pathway and NeuroInflammation: The Confused Brain in *Primer of PsychoNeuroImmunology Research* (ed. Opp, M. R.) 95–101 (2016).
3. Zhai L, Ladomersky E, **Dostal CR**, Lauing K, Swoap K, Billingham LK, Gritsina G, Wu M, M^{C}Cusker RH, Binder DC, Wainwright DA. Non-tumor cell IDO1 predominantly contributes to enzyme activity and response to CTLA-4/PD-L1 inhibition in mouse glioblastoma. *Brain, Behavior, and Immunity*. 62, 24-29 (2017).
4. **Dostal CR**, Carson Sulzer M, Kelley KW, Freund GG & M^{C}Cusker RH. Glial and tissue-specific regulation of *Kynurenine Pathway* dioxygenases by acute stress of mice. *Neurobiology of Stress.* 7, 1-15 (2017).
5. **Dostal CR**, Gamsby NS, Lawson MA, M^{C}Cusker RH. Glia- and tissue-specific changes in the *Kynurenine Pathway* after treatment of mice with lipopolysaccharide and dexamethasone.
   in Review

5.3 Other Research Projects *in prep*

6. **Dostal CR**, Braun JC, Gamsby NS, Kelley KW, M^{C}Cusker RH. Myeloid derived Ido1 contributes to inflammation induced anhedonia. (2017)
7. **Dostal CR**, Braun JC, M^{C}Cusker RH. Glucocorticoid and cytokine interactions regulate rate-limiting *Kynurenine Pathway* dioxygenases in murine astrocytes.
8. **Dostal CR**, Braun JC, Gamsby NS, Towers AE, Gainey SJ, Freund GG, Kelley KW, M^{C}Cusker RH. Neuron- and myeloid-derived Insulin-like growth factor (IGF)-I contributions to cognition and inflammation induced anhedonia of mice. (2017)

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